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Regulation, function, and evolution of T2 RNases

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ABSTRACT

T2 RNases have been identified in numerous organisms from plants to animals and even microorganisms. The distribution of this family in almost every organism suggests it may have an important biological function that has being conserved through evolution. In plants, two different subfamilies are defined. S-RNases are involved in pollen rejection during self-incompatible interactions, while S-like RNases are a more diverse group, with not clear function.

While expression studies suggest that S-like RNases are involved in many stress responses, including defense against pests and nutrient starvation, and in developmental processes such as senescence, functional studies addressing their biological role are still lacking. In an attempt to fill this gap in knowledge we initiated an analysis of RNS1, a RNase T2 enzyme from *Arabidopsis thaliana*. We showed that *RNS1* transcript and protein are induced during mechanical wounding of the plant and by treatment with the hormone Abscisic Acid (ABA). We found that ABA is part of the RNS1 wounding response pathway; yet in the absence of ABA the *RNS1* transcript is still induced. Thus, *RNS1* defines a novel wound-response pathway, independent of known wounding signals such as oligogalacturonides, jasmonates, and ethylene. The unusual regulation of *RNS1* by novel ABA-dependent and ABA-independent wounding response pathways suggest a unique, yet undefined, function.

To further study the function of T2 RNases, we extended our work to other organisms. We found that petunia nectar is rich in RNase activities, and we identified four T2 RNases in *Petunia hybrida*. Two of these RNases are similar to S-like RNases; while the other two contain characteristics similar to both S- and S-like RNases. The latter two (*RNase Phy3* and *RNase Phy4*) also show patterns of regulation consistent with those of nectarins; suggesting they may have a role in petunia nectar defense.

While expression analyses can provide clues to understand function of RNases, it was clear that the neither of these potential defense roles would be the one selected to keep this family in almost all organisms. Thus, we carried out

phylogenetic analyses in search of conservation patterns that could provide more information about this elusive biological role. To this end we characterized RNase T2 proteins from animals (zebrafish) and plants (rice) and identified RNase T2 genes from a variety of species with fully sequenced genomes. We identified two T2 RNase genes in the *Danio rerio* (zebrafish) genome. Patterns of regulation for these RNases suggest a possible housekeeping function. Evolutionary analysis of these enzymes along with the emergence of the RNase A family suggest many of the "stress" related functions preformed by T2 RNases in plants are carried out by the RNase A family in vertebrates; yet retention of at least one T2 RNase suggests an essential function exists.

Expression analysis of eight T2 RNases from *Oryza sativa* (rice) and phylogenetic analysis of plant T2 RNases present in other fully sequenced plant genomes to led us to conclude that plant S-like RNases are divided in two classes; with RNases in Class I showing signs of rapid evolution and a possible function in stress responses (defense, nutrient deficiency), whereas Class II RNases are expressed ubiquitously and phylogenic conservation suggests a possible housekeeping role. This housekeeping role may be conserved for RNase T2 proteins in animals, while Class I functions are carried out by RNase A proteins in vertebrates.

CHAPTER 1: General Introduction

Ribonuclease T2 – General Information

Ribonucleases (RNases) from the RNase T2 family are endoribonucleases located in cellular compartments of secretory pathway or secreted directly from the cell (Irie and Ohgi, 2001);(Deshpande and Shankar, 2002). The name T2 originated from an RNase of the fungi *Aspergillus oryzae* that was discovered by Sato and Egami (Sato and Egami, 1957), who proposed a mechanism for RNase T2 that released 3'-adenylic acid from RNA degradation. Later experiments demonstrated T2 family ribonucleases have no base preference. Enzymes of the T2 family have a molecular mass around 25kDa, no base specificity, and are considered to be acid RNases (Irie, 1999). A member of the T2 family of ribonucleases can be found in almost every organism examined including plants, fungi, bacteria, viruses, and animals (Deshpande and Shankar, 2002). This high rate of conservation throughout evolution suggests these ribonucleases have an important function in the organism. Due to gene duplication during evolution, many organisms have multiple T2 ribonucleases which have diverged to take on specialized functions (Taylor and Green, 1991).

In plants, the T2 family has been divided into the S-RNases and S-like RNases. Both S and S-like RNases are capable of degrading RNA through hydrolytic cleavage producing phosphomonoesters, yet their biological function is different. The S-RNases are involved in one selection process for gametophytic self-incompatibility (GSI). During this process, pollen lands on the stigma and pollen tube formation initiates towards the ovule. S-RNases, the female component of the GSI system, then penetrate the pollen tube. In a compatible interaction, when pollen and pistil do not share the same alleles, the S-RNase interacts with the pollen component, a ubiquitin E3 ligase called SLF, and the RNase is degraded. Pollen tube growth is not affected in this case. In incompatible interactions, where pollen and pistil have the same S-alleles, the interaction between S-RNase and SLF do not result in RNase degradation. The S-RNase is then able to degrade rRNA in the

pollen tube, which results in aborted pollen tube formation (Franklin-Tong and Franklin, 2003; Roalson and McCubbin, 2003). These S-RNases are typically localized to the style of the flower. RNase GSI is found in many of the plants in the *Solanaceae, Rosaceae, and Plantaginaceae* family. Two of the more highly researched plant genera with characterized S-RNases include *Nicotiana* and *Petunia*.

The S-like RNases, termed S-like because they bear high structural homology to the S-RNases and share conserved sequence patterns, are found in both self-compatible and self-incompatible plants. These RNases have certain amino acid motifs that are used to better categorize individual RNases into their respective classes. Despite their differences, both RNases have near identical amino acid motifs in their active sites supporting the notion that they arose from a common ancestor (Taylor *et al.*, 1993; Sassa *et al.*, 1996; Ushijima *et al.*, 1998). S-like RNases are expressed in many different organs including roots, leaves, stems, and flowers, whereas S-RNases are expressed in the pistil of the flower. The expression pattern for each individual RNase may vary alluding to specialized biological functions involving RNA degradation. S-like RNases are upregulated during periods of stress including phosphate limitation, wounding, pathogen infection, and during different developmental stages including senescence.

Expression Patterns of Plant S-like RNases

In tomato, two S-like RNases that accumulate during senescence have been well characterized. Expression analysis of *RNaseLE* and *RNaseLX* show induction of the mRNA transcript and protein during senescence (Lers *et al.*, 1998; Lers *et al.*, 2006). Both RNases are induced in tomato leaves and this expression can be artificially induced in leaves detached from the plant and incubated in the dark (Lers *et al.*, 1998). Basal levels of *RNaseLX* mRNA were detected in the roots, stems, and fruit at non-senescing time points. Immunodetection of proteins from abscission zone tissue during abscission has shown increased expression of RNaseLX; whereas tissue from a few millimeters away does not contain this enzyme (Lers *et*

al., 2006). In addition, plants expressing the anti-sense RNase*LX* gene have a delay in senescence and abscission (Lers *et al.*, 2006) suggesting RNaseLX is required for normal timing of abscission. In both abscission and senescence, ethylene is a key regulator. During ethylene treatment of senescing tomato plants, RNaseLX protein is upregulated in older more senescent leaves compared to leaves from comparable control plants (Lers *et al.*, 2006). Thus it is plausible to hypothesize that RNaseLX is an important enzyme needed for correct timing of abscission and senescence in tomato plants.

Tomato is not the only plant showing RNase induction during senescence. In Arabidopsis, the *RNS2* transcript is induced during senescence and those transcripts of *RNS1* and *RNS3* modestly (Taylor *et al.*, 1993; Bariola *et al.*, 1994). Low levels of *RNS2* mRNA are detected in the roots, stems, and leaves with the greatest expression occurring in the flowers (Taylor *et al.*, 1993). During senescence, a notable amount of increased transcript expression of *RNS2* is detected in the leaves. Thus induction of *RNS2* during leaf senescence is a noteworthy effect which correlates alongside the normal progression of senescence. In wheat, 3 RNases have been identified in senescent tissue (Blank and McKeon, 1991). The protein activity of these RNases was identified in both natural and dark-induced senescing tissue and suggests they may have a unique function during senescence (Blank and McKeon, 1991).

Analysis of expression patterns suggest that the S-like RNases are also involved in phosphate starvation (Taylor *et al.*, 1993; Hayashi T, 2003; Kock *et al.*, 2006). During growth in phosphate limitation, plants will implement strategies for conservation of phosphate. Such strategies often include scavenging for Pi (inorganic phosphate) from cellular macromolecules and nutrients in their immediate environment (ie: soil). These macromolecules often include nucleic acids such as RNAs whose degradation could be part of the Pi deficiency response in plants (Kock *et al.*, 2006). In Arabidopsis, *RNS1* and *RNS2* transcript levels are induced during phosphate limitation (Taylor *et al.*, 1993; Bariola *et al.*, 1994). *RNS2* is present at detectable levels prior to phosphate limitation studies unlike the *RNS1* mRNA which

is not expressed prior to the treatment. This suggests that *RNS2* expression is not regulated solely based on Pi limiting conditions; however *RNS1* is regulated by Pi conditions. Another RNase, *RNS3* has a great similarity to *RNS1* but its transcript level remained unaffected during the response to Pi-starvation (Taylor *et al.*, 1993).

Accumulation of RNaseLX and RNaseLE proteins is induced in tomato cell cultures grown under Pi limiting conditions (Loffler *et al.*, 1992), (Nurnberger *et al.*, 1990) (Kock *et al.*, 1995). These RNases are upregulated not only in cell cultures but also in planta (Kock *et al.*, 1995; Kock *et al.*, 2006). In cell cultures, expression of the RNases was upregulated during Pi starvation and their transcript levels decreased when phosphate was supplemented back into the media thus demonstrating their regulation by Pi limiting conditions (Kock *et al.*, 1998). The use of transgenic plants containing the Gus reporter gene under the control of the *RNaseLX* promoter showed inducible expression during Pi starvation in primary and lateral root tips. This expression correlated with an increase in root growth during starvation conditions; however the *RNaseLX* transcript was not induced by auxin or ethylene treatments, although these hormones have been found to regulate root growth (Kock *et al.*, 2006). Thus, expression of these RNases is regulated independently of these hormones during phosphate starvation conditions.

Another S-like RNase known as *RNaseNE* from *Nicotiana alata* also shows regulation during Pi limiting conditions (Dodds *et al.*, 1996). The amino acid sequence of RNaseNE from tobacco is 86% similar to RNaseLE from tomato. Detection of the *RNaseNE* transcript is limited to some reproductive tissues and is not found in vegetative tissue under normal growth conditions; however *RNaseNE* mRNA is expressed in the roots, but not in vegetative tissue, during a 12 day Pi limitation experiment suggesting its expression is regulated by Pi starvation conditions (Dodds *et al.*, 1996).

RNase *AhSL28* mRNA from *Antirrhinum* also increases during phosphate starvation treatments (Liang *et al.*, 2002). Phylogenic analysis of AhSL28 shows it is most similar to RNS2 from *Arabidopsis* (63% similarity) in amino acid sequence and

genomic structure (Liang *et al.*, 2002). Besides being induced during Pi starvation conditions its transcript is also regulated during leaf senescence (Liang *et al.*, 2002).

S-like RNases are also upregulated in response to wounding. In *Arabidopsis*, the *RNS1* transcript is induced locally and systemically in response to mechanical wounding (LeBrasseur *et al.*, 2002). Induction of the *RNS1* gene is independent of the known wounding pathways in *Arabidopsis* which use Jasmonic Acid (JA) and oligogalactorunides (OGA) as signaling molecules. Treatment of *Arabidopsis* plants with JA and OGA failed to induce the *RNS1* transcript; likewise *RNS1* expression was still observed in plants with impaired JA signaling. In addition, mutant plants with other impaired hormone pathway genes such as *ein2* and *NahG* plants still express *RNS1* mRNA during wounding (LeBrasseur *et al.*, 2002). Thus leading to the proposal of an alternative wounding pathway through which *RNS1* mRNA is induced.

In tomato, *RNaseLE* mRNA and protein are also induced in response to wounding (Kock *et al.*, 2004). Unlike *RNS1* in *Arabidopsis*, *RNaseLE* expression is induced only locally during the wounding response. Wound response pathway signaling molecules such as JA, OGA, ethylene, ABA, and salicylate failed to induce *RNaseLE* (Groß *et al.*, 2004). To further test expression of *RNaseLE* independent of the JA pathway, *AOC* (allene oxide cyclase) *sense* and *antisense* mutants were used. These mutants are highly responsive to JA or insensitive, respectively. In both experiments expression and activity of *RNaseLE* remain unchanged from that of a wild-type plant (Groß *et al.*, 2004). These results suggested RNaseLE may be part of a wound healing process or part of the apoptotic process near wound sites (Groß *et al.*, 2004).

Tobacco *RNaseNW* is also induced in response to wounding of the leaves (Kariu *et al.*, 1998). Increase of *RNaseNW* transcript level induced by wounding can be detected in as little as 4 hours and gradually decreases during 20 hours (Kariu *et al.*, 1998). When wounded, *Zinnia elegans* also induces the transcript of an S-like ribonuclease known as *ZRNaseII* (Ye and Droste, 1996). This RNase shows no expression in other tissue prior to the induction response during wounding. At the

protein level, ZRNaseII is similar to RNaseNW and RNaseLE, two other wound inducible ribonucleases. Though they are induced during the wounding response, there are still questions about the regulation of these enzymes and their function in this process.

RNS1 in *Arabidopsis* is induced at both the transcript and protein level not only by the wounding response pathway but also during phosphate starvation conditions. Analysis of gene expression of *RNS1* revealed it is upregulated independently of the known wounding pathways in *Arabidopsis* making it an intriguing protein to study regarding regulation and function.

S-like RNases are also induced during pathogen attack. In *Nicotiana glutinosa,* the *NGR3* gene was found to be induced 48 hours after tobacco mosaic virus (TMV) infection unlike two other RNases from tobacco (*RNaseNW* and *NGR2*) (Kurata *et al.*, 2002); (Hayashi T, 2003). When TMV infection was preceded by wounding, induction of *NGR3* was noted 48-72 hours following treatment (Kurata *et al.*, 2002). This specific induction due to TMV infection suggests *NGR3* may play a specific role in plant defense during viral infection.

Expression of another ribonuclease from tobacco plants, *RNaseNE,* was shown to be upregulated when tobacco plants were challenged with the fungi *Phytophthora parasitica (P. parasitica)* (Galiana *et al.*, 1997). Exogenous application of RNase A, a vertebrate-specific secreted RNase with activity similar to T2 enzymes, to the tobacco leaves resulted in a decrease in the development of the fungus by 90% (Galiana *et al.*, 1997). During fungal tip growth of *P. parasitica* into the apoplastic space of the tobacco leaves, antibodies to RNaseNE confirmed this protein was indeed secreted from the cells (Hugot *et al.*, 2002). Additionally, application of exogenous RNaseNE protein to tobacco leaves inhibits hyphal development of the fungus in the apoplastic space (Hugot *et al.*, 2002) supporting the idea that regulation of RNaseNE during microorganismal infection is part of a defense mechanism. In addition, purified RNaseNE protein was able to inhibit *Fusarium oxysporum* conidia *in vitro* (Hugot *et al.*, 2002). Although it appears that

some S-like RNases participate in defense responses their role in these responses is not clearly understood.

Differences in S- and S-like RNases

S and S-like RNases may have similar protein structures and conserved active sites, yet their tissue specificity and biochemical properties are very different. S-RNases are typically expressed in the stigma or style of the flower; whereas most S-like RNases are expressed in all tissues of the plant. These tissues include not only the stigma or style of the flower, but also petals, anthers, leaves, phloem, and roots. Tissue-specific expression varies depending on the RNase being studied. *RNS2* and *NGR2* transcripts are expressed ubiquitously throughout the plant. Contrastingly, *RNS1* and *RNaseNW* transcripts are not detected in unwounded vegetative tissue (LeBrasseur *et al.*, 2002) (Kariu *et al.*, 1998). *RNaseLE* mRNA is expressed in undeveloped tracheary elements, senescing flowers and leaves, and in the endosperm during germination (Lehmann *et al.*, 2001). Tissue-specific expression of S-like RNases suggest each one has a unique function in the plant.

Biochemically, S- and S-Like RNases are also somewhat different. S-RNases are basic glycoproteins (McClure *et al.*, 1989) with a pI range of 7-8. S-like RNases are typically acidic proteins with a pI range of 4.0-5.0 (Irie, 1999) and function better *in vivo* in a neutral or acid environment. Some S-like RNases are glycosylated, while others are not.

Phylogenetic alignments of T2 RNases group these RNases into 3 main classes that coincide with differences in the copy number and location of introns in the genomic structure. Class I contains non-S RNases with less than 4 introns and have multiple copies within the genome. This class includes *RNS1, RNS3, RNaseLE, RNaseLX,* and *RNaseNW* for example (Igic and Kohn, 2001). Class II RNases are non-S RNases present only in a single copy in the genome and have more than 4 introns, typically 7 or 8. These RNases also have a conserved disulfide bridge at the N-terminal end of the protein that is not present in the other classes, and include *RNS2*, tobacco *RNase NGR2*, and tomato *RNase LER2* (Igic and Kohn,

2001). The third and final class, Class III, contain the typical S-RNases with one or two introns present. This class includes all the RNases involved with self-incompatibility and some of the more recently identified relic RNases. Additionally, unique protein motifs have been used as a mechanism to determine the probability of a T2 RNase belonging to either the S- or S-like subfamily (Vieira *et al.*, 2008).

T2 RNases in Animals

Orthologues of T2 RNases have been found in organisms beside plants. In lower eukaryotes, the orthologue known as *RNY1* (Rny1p) was identified in *Saccharomyces cerevisiae* (MacIntosh *et al.*, 2001). This species contains only one copy of a T2 RNase. Mutant lines where this secreted enzyme was inactivated obtained a unique phenotype. Recombinant yeast became enlarged and developed temperature sensitivity (MacIntosh *et al.*, 2001), although the phenotype is strain-specific. This phenotype led to the proposal that RNases may alter membrane stability and permeability (MacIntosh *et al.*, 2001). Recently, Rny1p has been shown to cleave tRNA during periods of oxidative stress and localize to the vacuole (Thompson and Parker, 2009). During hydrogen peroxide treatment, this RNase is released from the vacuole and cleaves tRNA only in the affected cell and not from a neighboring cell (Thompson and Parker, 2009). Overexpression of Rny1p created a cell line which had a reduced viability during oxidative stress treatment; suggesting Rny1p overexpressors had hypersensitivity to oxidative stress (Thompson and Parker, 2009). Together these results suggest Rny1p is an important component of the yeast stress response.

Recently a human T2 RNase was identified. Human *RNASET2* is the first T2 RNase glycoprotein identified in humans (Campomenosi *et al.*, 2006). RNASET2 protein is capable of binding actin, inhibiting the clonogenicity (cancer cell colony formation)of HT29 cells, and reducing the size of tumors in nude mice (Smirnoff *et al.*, 2006). The actin binding and anti-clonogenic properties possessed by RNASET2 are independent of its RNase activity. This independence has been shown for another RNase, ACTIBIND, also a member of the RNase T2 family (Roiz

et al., 2006). RNASET2 has also been linked to the suppression of oncogenesis in melanoma (Monti et al, 2008) and ovarian cancer cell models (Acquati *et al.*, 2005). Transfected melanoma cells which contained RNASET2 were injected into nude mice models and tumor size was measured. Mice with RNASET2 tumors had a significantly lower growth rate than the control tumor mice (Monti *et al.*, 2008). Although RNASET2 is similar in sequence to RNY1 from yeast, it is unable to function in the tRNA cleavage during oxidative stress demonstrating evolution of ribonuclease enzymes acquiring functions held by T2 RNases in other systems (Yamasaki *et al.*, 2009).

In addition, S-like RNases have also been identified in higher eukaryotes such as cow, chicken, and fish (Irie, 1993; (Hillwig *et al.*, 2009); (Trubia *et al.*, 1997). In some cases the protein was purified from a specific organ or tissue and in other cases the proteins were generated from cDNA clones. Unlike the plant S-like RNases, little is known about the regulation or function of animal T2 RNases.

RNase A Family

In animals an additional distinct RNase family known as the RNase A family exists. RNase A has been well characterized; chronologically being the third protein, and first RNase, to have its crystal structure solved (Wyckoff *et al.*, 1967). RNase A proteins have characteristics similar to those noted in the T2 RNase family. The RNase A family is a vertebrate specific RNase family found in mammals, birds, reptiles, amphibians and some species of bony fish. They are extracellular proteins which have cytotoxic effects, and antimicrobial properties.

In humans, an angiogenin RNase known now as RNase hANG, was the first human derived tumor protein found to have angiogenic ability *in vivo* (Fett *et al.*, 1985). Injection of this protein was able to stimulate new blood vessel growth in the eye of a rabbit demonstrating the first case of an RNase with angiogenic abilities (Fett *et al.*, 1985). However, when this enzyme is inhibited with an RNase inhibitor, RNase activity and angiogenic properties are also abolished (Shapiro and Vallee, 1987). Additional research on hANG showed it was also able to cleave tRNAs

(Saxena *et al.*, 1992). The degradation of tRNAs is an example where one of the functions of a T2 RNase (Rny1p) has been taken over by an RNase A homolog in a higher eukaryote.

Cellular Localization of RNase T2 Proteins

Alongside studying regulation to understand function of ribonucleases, determining localization of these specialized enzymes may help to better predict their function. S-like RNases are expressed in different plant tissues; yet their cellular location is relatively consistent. All known S-like RNases have an N-terminal signal peptide motif, which directs the enzyme to the secretory pathway. Some RNases also contain additional signals which allow them to be retained in a component of the secretory pathway such as the ER, vacuole, or lysosome.

RNaseLE protein from tomato is retained in the vacuole of cell culture lines (Loffler *et al.*, 1992) and released from cells in Pi starvation cell culture experiments (Nurnberger *et al.*, 1990) yet it has no discernible vacuolar localization signal. RNaseLX contains a C-terminal peptide HDEF which targets the protein to the ER (Lehmann *et al.*, 2001). Likewise the NGR3 RNase from tobacco contains the same HDEF peptide suggesting it may also be retained in the ER (Kurata *et al.*, 2002). Recently an RNase from *Bacillus cereus* was isolated and shown to inhibit tobacco mosaic virus (Zhou and Niu, 2009). This RNase is extracellular and secreted from the bacterium suggesting it may be involved in a mechanism of plant pathogen defense.

In animals RNase localization has also been studied. RNASET2 from humans has been shown to have a lysosomal localization which is consistent with the localization of other members of the T2 RNase family (Campomenosi *et al.*, 2006). Yeast cells secrete the Rny1p ribonuclease from the cell into the media as shown by in gel assays (MacIntosh *et al.*, 2001), and also accumulate in the vacuole (Thompson, et al 2009).

Conclusion

Secretory RNases are important enzymes with functions and regulations not well characterized. Plant RNase T2 proteins are involved in many stress responses. Regulation by Pi-starvation and senescence suggest that these enzymes are part of a phosphate-scavenging system. Expression of these genes in response to pathogens and wounding indicates a role in defense responses, although the actual biological role in these responses is not well understood. In contrast to plants, vertebrates lack multiple T2 RNases and we hypothesize that RNase T2 diversification with regards to function may have been prevented by when the RNase A family appeared. In this dissertation we aim to extend the knowledge of the function, regulation, and localization of T2 RNases that will allow a better understanding of the biological role of this RNase family.

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CHAPTER 2: Impact of transcriptional, ABA-dependent, and ABA-independent pathways on wounding regulation of *RNS1* expression

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Authors' contributions

MSH carried out the gene expression analyses, RNase activity characterization in each treatment, and photographs of the GUS/luciferace plants. NDL performed the initial ABA expression analysis, identified elements in the promoter region, and made all the constructs used for this study. PJG and GCM conceived of the study. MSH and GCM participated in the design of the study and drafted the manuscript. All authors read and approved the final manuscript.

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Abstract

Injured plants induce a wide range of genes whose products are thought to help to repair the plant or to defend against opportunistic pathogens that might infect the wounded plant. In *Arabidopsis thaliana* L., oligogalacturonides (OGAs) and jasmonic acid (JA) are the main regulators of the signaling pathways that control the local and systemic wound response, respectively. RNS1, a secreted ribonuclease, is induced by wounding in Arabidopsis independent of these two signals, thus indicating that another wound-response signal exists. Here we show that abscisic acid (ABA), which induces wound-responsive genes in other systems, also induces RNS1. In the absence of ABA signaling, wounding induces only approximately 45% of the endogenous levels of *RNS1* mRNA. However, significant levels of RNS1 still accumulate in the absence of ABA signaling. Our results suggest that wound-responsive increases in ABA production may amplify induction of RNS1 by a novel ABA-independent pathway. To elucidate this novel pathway, we show here that the wound induction of *RNS1* is due in part to transcriptional regulation by wounding and ABA. We also show evidence of post-transcriptional regulation which may contribute to the high levels of *RNS1* transcript accumulation in response to wounding.

Key words: abscisic acid, post-transcriptional regulation, promoter, ribonuclease, wounding

Abbreviations:

ABA: abscisic acid
ABRE: ABA-responsive element
DRE: dehydration response element
JA: jasmonic acid
OGA: oligogalacturonides
RNase: ribonuclease
SA: salicylic acid

Introduction

Secreted ribonucleases (RNases) are enzymes located where RNA is not presumed to be readily available, such as in the vacuole or outside the cell. The T_2 superfamily of secreted RNases, in particular, has been found in nearly every system examined for their presence, including fungi, viruses, bacteria, plants, and animals (Deshpande and Shankar 2002). The ubiquitous distribution of T_2 RNases suggests they have both an ancient origin and critical function(s) (Taylor and Green 1991).

Despite the apparent necessity for the activity of T_2 enzymes, very little has been demonstrated regarding their biological functions. The exception is S-RNases, a class of plant T_2 RNases whose activity is essential for the process of self-incompatibility in several plant families (reviewed in McCubbin and Kao 2000). Enzymes related to, but distinct from, S-RNases are also present in self-compatible plants and form a class known as S-like RNases (reviewed in Bariola and Green 1997). S-like enzymes are not involved in self-incompatibility, but seem to have important functions throughout the plant kingdom, as they are ubiquitous in plants. The *Arabidopsis thaliana* genome contains five S-like genes, *RNS1* to *RNS5* (Taylor and Green 1991; G.C. MacIntosh, unpublished), and RNase activity has been demonstrated for the products of three of these (Taylor et al. 1993; Bariola et al. 1994).

Fluctuations in RNase activity levels or gene expression are useful for predicting RNase function. The discovery that growth on low concentrations of inorganic phosphate (P_i) induces expression of various RNases, including Arabidopsis RNS1 and RNS2 (Bariola et al. 1994, 1999) and tomato RNases LX and LE (Nürnberger et al. 1990; Bosse and Köck 1998), led to the hypothesis that S-like RNases are part of a rescue system that plants use to recycle P_i when environmental pools are limiting (Goldstein et al. 1989).

In addition to low inorganic phosphate concentration, RNases are also induced by wounding in several plant systems. For example, the transcript for RNase LE accumulates in wounded tomato leaves (Lers et al. 1998; Groß et al. 2004), and RNase NW is induced in wounded tobacco leaves (Kariu et al. 1998). We showed that RNS1 and several nuclease activities are coordinately regulated by wounding in Arabidopsis (LeBrasseur et al. 2002). The *RNS1* transcript was

the most highly wounding-induced transcript in two independent microarray experiments – one examined 150 genes enriched for those implicated in defense responses (Reymond et al. 2000), and the second examined 600 genes, about half of which were hypothesized to be involved in RNA metabolism and turnover (Pérez-Amador et al. 2002). The strong *RNS1* transcript accumulation may indicate that RNS1 has a critical function during wounding. RNS1 transcript and activity is also increased in non-damaged tissue of wounded plants, where recycling of nutrients and degradation of bulk cellular nucleic acid, should not be necessary. We therefore proposed that RNS1 may also have a defensive function (LeBrasseur et al. 2002).

The induction of RNS1 and nuclease activities provides us with a unique perspective into Arabidopsis wound signaling mechanisms (LeBrasseur et al. 2002). Our understanding of the wound response in Arabidopsis is currently highlighted by the presence of two distinct, antagonistic pathways: JA-dependent and -independent. The JA-independent pathway controls local transcript accumulation and has been shown to be regulated by OGA elicitors probably released from injured plant cell walls (Rojo et al. 2003).

Although RNS1 and the three nuclease activities are strongly induced locally by wounding, they are not induced by treatments with OGA-rich fractions (LeBrasseur et al. 2002). The local response of RNS1 and the nucleases to wounding is also not controlled by the JA-dependent signaling pathway, as shown by the strong wound-induction of these activities in the JA-insensitive *coi1* mutant. It has been proposed that JA signaling controls the systemic wound response in Arabidopsis (Titarenko et al. 1997; León et al. 2001) but the systemic induction of *RNS1* did not depend on JA (LeBrasseur et al. 2002). To our knowledge, *RNS1* was the first gene in Arabidopsis shown to be induced systemically by wounding in a JA-independent manner and therefore indicates the existence of an alternate long-distance signaling pathway.

It is becoming clear that JA-independent pathways are important in the regulation of wounding response, however very little is known about the signal transduction pathways controlling these responses (Howe 2004). Several molecules have been proposed to act as signals in the wounding response in plants in addition to OGAs and JA (reviewed in de Bruxelles and Roberts 2001;

León et al. 2001; Howe 2004), including abscisic acid (ABA, see review by Lorenzo and Solano 2005). ABA application induces the local and systemic expression of *PinII*, a wound-inducible gene, in potato, tomato and tobacco (Peña-Cortés et al. 1989). Analyses of ABA-deficient mutants of potato and tomato provided further evidence for a requirement for ABA in the wound-induction of *Pin* genes (Peña-Cortés et al. 1989; 1991), and ABA accumulates upon wounding (Peña-Cortés et al. 1991). However, the role of ABA in the wounding response is controversial. Birkenmeier and Ryan (1998) found that exogenous ABA induces *PinII* expression in tomato to a much lesser extent than either wounding or JA application and that endogenous ABA levels only increase significantly at the wound site.

Recent evidence suggests that *RNS1* may be controlled by ABA signaling. A mutant screen identified an mRNA cap-binding protein, ABH1, as a negative modulator of ABA signaling in stomata (Hugouvieux et al. 2001). DNA chip analyses comparing gene expression in WT and *abh1* plants identified *RNS1* as one of a few transcripts that are down-regulated in *abh1.* These genes might function in early ABA signaling, as their transcripts represent putative targets for ABH1-dependent mRNA processing (Hugouvieux et al. 2001). As ABA is a proposed regulator of the wounding response in other plants, it could also control the OGA- and JA-independent pathway defined by *RNS1* expression in Arabidopsis. In addition, the *abh1* results indicate that ABA might post-transcriptionally stabilize *RNS1* mRNA after wounding. Here we show that ABA induces *RNS1* expression with a timing that is similar to that of wounding. We also show that ABA is necessary to produce the full wounding response. However, ABA is only part of the signaling pathways controlling RNS1 induction in wounded Arabidopsis plants. Our results indicate that an as-yet uncharacterized ABA-independent pathway, independent of JA and OGA as well, also contributes to *RNS1* induction during the wounding response. We found evidences that this novel pathway acts synergistically with ABA to regulate *RNS1* induction at the transcriptional level. The possibility of post-transcriptional regulation is also discussed.

Materials and Methods

Plant materials and treatments

Unless otherwise stated, the Columbia-0 ecotype of *Arabidopsis thaliana* L. was used throughout this study. Soil-grown plants were grown in chambers under 16 h of light in 50% relative humidity at 20°C. For seedling experiments, seeds were surface-sterilized and germinated on Arabidopsis growth medium as described (Taylor et al. 1993). The *aba1-1, abi1* and *abi2* seeds were kindly provided by Dr. Michael Thomashow (Michigan State University), *abi4* and *abi5* seeds were obtained from the Arabidopsis Biological Resource Center (ABRC). For wounding treatments, leaves of 4- to 6-week-old plants or leaves of 14-day-old seedlings were wounded using ridged flat-tipped tweezers, harvested at subsequent timepoints, and treated as previously described (LeBrasseur et al. 2002). ABA treatments were conducted on 14-day-old seedlings grown on MS-agar plates covered with plastic mesh. Seedlings were transferred to 0.5× MS medium (Sigma, Saint Louis, MO) with or without 100 μM ABA (Sigma, Saint Louis, MO) and harvested at subsequent timepoints. WT controls for the ABA mutant experiments were performed with the ecotype Landsberg erecta (Ler), Columbia-0 (Col) or Wassilewskija (Ws) as indicated. Experiments were performed a minimum of three times. Representative blots or gels are shown.

Plants were transformed by vacuum infiltration as previously described (Bariola et al. 1999). For each experiment, at least 3 independently transformed lines were used. Representative results are shown.

Cloning and sequence analysis

Standard cloning techniques were used throughout. The *RNS1* promoter region was isolated previously (Howard 1996) and contains 2.6 kb of DNA upstream of the *RNS1* initiation codon. This includes DNA from chromosome 2 coordinates 870957 (5') to 873663 (3'), based on the current AGI annotation as shown at TAIR (www.arabidopsis.org), which corresponds to the TAIR 7 version of the Arabidopsis genome, released in April 2007. The promoter region was cloned into a Bluescript II vector (Stratagene, La Jolla, CA) containing the β-glucuronidase (GUS) protein sequence (Jefferson et al. 1987) with a *rbcS-E9*

polyadenylation sequence (Fang et al. 1989). We removed the short stretch of 5' UTR sequence that was present in this construct to create a 3' end on the *RNS1* promoter that corresponds to coordinate 873563. This construct was then cloned into an *Agrobacterium tumefaciens* shuttle vector containing a kanamycin resistance gene as described before (Gil and Green 1996) and designated p2081. In plasmid p2082 the GUS coding region was replaced by *luciferase* (Millar et al. 1992). Construct p848 (35S-GUS-E9), containing the cauliflower mosaic virus 35S promoter in place of the *RNS1* promoter, was constructed in a similar manner (Howard 1996).

The *nos* (nopaline synthase) promoter was amplified by PCR from pBI-121 using the primers PG-454 (5'-gatcatctgcagagaattaagg) and PG-453 (5'-gttcaaccatgggaaacgatcc). The nos-globin-E9 construct was made by replacing the 2x35S promoter of p1185 (Diehn et al. 1998) with *nos* to make p2031. The *RNS1* cDNA (Bariola et al. 1994) was then inserted in place of *globin* to make p1966. The entire nos-RNS1-E9 cassette was then cloned into the plant transformation vector pCambia 1301 (GenBank accession number AF234297), which has the hygromycin resistance plant selection marker. This clone was named p1975. The nos-globin-E9 cassette was cloned into pCambia 2301 (GenBank accession number AF234316), which confers kanamycin resistance to transformed plants, and was named p1995. The entire *RNS1* transcribed region, including the full 5' UTR and introns, was PCR-amplified. PCR products were sequenced to assure no errors were introduced and then inserted in place of *globin* in p2031. The orientation of the insert was confirmed and then the nos-preRNS1-E9 cassette was ligated into pCambia 2301.

Computational analysis of the proximal 1000 nt of the promoter sequence was performed using two internet-accessible databases, PlantCARE (Lescot et al. 2002) and PLACE (Higo et al. 1999). Only elements in which the core is absolutely conserved are reported here.

RNA and protein extraction and analysis

Total RNA from Arabidopsis samples was extracted and analyzed as previously described (LeBrasseur et al. 2002). RNA blots were hybridized using a ^{32}P-labeled *RNS1* probe. To control for loading, the same RNA blots were

stripped and then hybridized with a ^{32}P-labeled probe for the Arabidopsis translation elongation factor *EF-1α* (EST accession number R29806) or translation initiation factor *eIF-4A* (Taylor et al. 1993). The *COR6.6* gene, kindly provided by Dr. Michael Thomashow (Michigan State University), was used as a positive control for ABA treatments (Hajela et al. 1990). *GUS* and *globin* probes were prepared by PCR. The *nos* probe was prepared by polynucleotide kinase end-labeling of an antisense oligonucleotide using ^{32}P-ATP (sequence: GATCCAGATCCGGTGCAGATTATTTGGATTGAGAGTGAATAT). All blots were exposed for 16 hs, except RNS1p-GUS constructs that were exposed for 3-5 days. Blots were quantified using PhosphorImager. *RNS1*, *GUS* and *nos* expression data were normalized using *EF-1α* values (as *RNS1*/*EF-1α*; *GUS*/*EF-1α; nos/ EF-1α*). The ratios from at least 3 independent experiments were used for the expression data shown in figures 2b, 5b, 6c and 6d. For the nos-RNS1 constructs, both the individual bands and the doublet as a whole were quantified. Results for the doublet are reported, but individual bands gave similar results.

To verify the identity of the two bands obtained with the nos-RNS1 reporter constructs, 3' rapid amplification of cDNA ends (RACE) was performed using the 3' RACE System (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. Gene specific primer for initial PCR was the *nos* probe describe above. Primers for nested PCR were the GTGTTTGATCAGTCTTCTCGTAATCTTGC (RNS1) and CTGATGCATTGAACTTGACGAACGTTGTCG (E9).

Total protein was extracted and RNase activities were assayed as described previously (LeBrasseur et al. 2002). Equal loading of protein gels was confirmed by Coomassie Blue staining of standard SDS-PAGE loaded with the same volume of protein extracts used for activity assays. All blots and gels are representative of at least three independent experiments.

Histochemical GUS staining and luciferase imaging

Histochemical localization of GUS activity was determined using a β-Glucuronidase Reporter Gene Staining Kit (Sigma) according to manufacturer's recommendations. Luciferase activity was analyzed using a CCD camera (ChemiPro System, Roper Scientific, Trenton, NJ) as described by Chinnusamy et al. (2002); exposure time was 20 min.

Results

RNS1 expression is induced by ABA

It has been suggested that ABA may regulate *RNS1* transcript accumulation, as loss of an mRNA-binding protein, ABH1, that downregulates ABA responses leads to reduced *RNS1* transcript levels (Hugouvieux et al. 2001). Transcriptional regulation of gene expression by ABA has been characterized to a large degree. Thus, we analyzed the *RNS1* promoter sequence to identify putative regulatory elements (Figure 1a and supplemental figure S1). A search for regions with homology to known regulatory elements identified three putative ABA-responsive elements (ABREs; Yamaguchi-Shinozaki and Shinozaki, 1993, 1994), and one MYB- and three MYC-binding regions. Some members of the MYC and MYB transcription factor families are induced by drought and ABA (Abe et al. 1997). A dehydration response element (DRE) was also found in the *RNS1* promoter. This element has been shown to be sufficient for a rapid response to dehydration without the involvement of ABA (Yamaguchi-Shinozaki and Shinozaki, 1993, 1994). In addition, several wounding-responsive elements were found in the *RNS1* promoter: a W-box (Eulgem et al. 1999) and two WUN elements (Pastuglia et al. 1997).

To test whether ABA could in fact induce *RNS1* expression, we treated Arabidopsis seedlings with ABA and samples were harvested at different time points. Mock-treated plants were harvested as control. Figure 1b shows that *RNS1* is induced in seedlings treated with ABA with kinetics similar to that of *COR 6.6,* a known ABA regulated gene (Gilmour et al. 1992). Using the ABA-insensitive mutant *abi2* (see below) we also showed that the induction of *RNS1* by ABA is regulated by the ABI2 pathway (Supplemental figure S2a). In the *abi2* mutants ABA is unable to induce *RNS1* accumulation.

We also analyzed the induction of RNS1 activity by ABA using an *in gel* activity assay. In this assay extracts are resolved by semi-denaturing SDS-PAGE using gels containing RNA, later incubated in activity buffer, and finally stained to detect RNA. Clear bands represent ribonuclease activities (Supplemental figure S2b). Twelve hours after ABA treatment an increase in RNS1 activity is clearly

observed, and it maintains similar levels after 24 hours. Thus, ABA is able to induce *RNS1* mRNA accumulation followed by an increase in RNS1 activity.

Both ABA-dependent and -independent pathways control *RNS1* induction by wounding

Because *RNS1* expression is induced by ABA, we tested whether ABA is the signal that controls the wounding pathway resulting in the accumulation of *RNS1* transcript and protein. To address this question, we took advantage of a series of mutants deficient in ABA production and signaling. *ABI1* and *ABI2* encode protein phosphatases that participate in the transmission of the ABA signal (Leung et al. 1994; 1997). Mutant plants carrying the *abi1* and *abi2* alleles are insensitive to ABA. In addition, *aba1-1* mutant plants possess a non-functional zeaxanthin epoxidase and cannot produce ABA (Rock and Zeevaart 1991); consequently, ABA-dependent processes are inhibited in these plants.

RNA blot analyses revealed that the wounding induction of *RNS1* transcript accumulation in *abi1*, *abi2*, and *aba1-1* mutants is only 32-48% that of the WT plants (Figures 2a and 2b). Our results indicate that an ABA-dependent pathway is required for the full induction of *RNS1* after wounding. However, wounding still induces *RNS1* expression in these mutants; thus, an as-yet uncharacterized ABA-independent pathway is responsible for the induction of *RNS1* in the absence of ABA signaling. As described previously (LeBrasseur et al. 2002), this pathway is also independent of JA and OGA, the two signals commonly associated with wounding responses in Arabidopsis.

We also analyzed the role of ABA on the wound-dependent increase in RNS1 activity by *in gel* RNase activity assay. Figure 2c shows that the increase in RNS1 activity observed after ABA treatment is absent in the *abi2* mutant. In addition, a modest decrease in activity (although this assay is not truly quantitative we estimated a reduction of ~25%) can be observed in wounded *abi2* plants with respect to wounded WT plants. These results are similar to those obtained by northern blots, and confirm the existence of two pathways that control the expression of *RNS1* and the increase in RNS1 activity in response to wounding. RNS1 induction by wounding is paralleled by an increase in several nuclease activities that degrade both RNA and DNA (LeBrasseur et al. 2002). Both the sustained induction of the 33-kD activities and the transient increase in

the 35-kD activity still occur in all the tested ABA mutants (data not shown), indicating that the uncharacterized ABA-independent pathway is also at least partially responsible for the induction of other nuclease activities after wounding.

In an initial attempt to dissect the ABA-dependent pathway controlling *RNS1* expression we analyzed whether any of the most common transcription factors involved in regulation of ABA-dependent transcription was necessary for wound induction of *RNS1*. Three different classes of transcription factors have been identified through genetic screenings of plants with reduced sensitivity to ABA (reviewed by Finkelstein et al. 2002). The *abi3* mutation corresponds to a B3-domain transcription factor (Giraudat et al. 1992), while *abi4* and *abi5* correspond to APETALA2 domain (Finkelstein et al. 1998) and bZIP domain (Finkelstein and Lynch 2000) transcription factors respectively. Microarray analyses indicate that ABI3 does not control *RNS1* expression (Suzuki et al. 2003). Thus, we tested whether ABI4 or ABI5 were responsible for ABA-dependent induction of *RNS1*. WT and mutant *abi4* and *abi5* plants were wounded and RNA was extracted 4 hours later. We found that neither ABI4 nor ABI5 are necessary for full induction of *RNS1* by wounding (Supplemental Figure S3). These results suggest that another transcription factor is responsible for ABA-dependent induction of *RNS1* by wounding. Alternatively, post-transcriptional processes could be invoked to explain this regulation.

Evidence for transcriptional and post-transcriptional control of *RNS1* by wounding and ABA

As a first step toward dissecting the regulatory mechanisms that control *RNS1* gene expression, we investigated whether *RNS1* transcript accumulation is controlled at the transcriptional and/or the post-transcriptional level. To this end we made transgenic Arabidopsis lines carrying the constructs depicted in Figure 3. Transcriptional regulation was analyzed using the construct RNS1p-GUS (Figure 3b), in which a 2.6-kb fragment corresponding to the *RNS1* promoter region controlled the expression of the *β-glucuronidase* (*GUS*) reporter gene. The same reporter driven by the CaMV *35S* promoter was used as control (35S-GUS; Figure 3a). Transformed plants were analyzed by RNA gel blots.

As shown in Figure 4, *RNS1* is regulated at the transcriptional level. In untreated leaves of plants transformed with the RNS1p-GUS construct, the *GUS* transcript is not detected. But 4 h after wounding, the *GUS* transcript is clearly expressed in wounded leaves. The control 35S-*GUS* lines showed no response to wounding. Similarly, *GUS* accumulation is also observed in plants treated with ABA. These results show that the *RNS1* promoter is sufficient to provide a transcriptional response to wounding and ABA.

Although endogenous levels of *RNS1* transcript are induced both by wounding and ABA, after 4 h endogenous *RNS1* expression is five-fold higher in wounded plants than in ABA-treated plants (Figure 5a and 5b). Side-by-side comparison of the levels of *GUS* reporter transcript showed that *GUS* expression is similar or higher in RNS1p-GUS plants treated with ABA compared to those that were wounded (Figure 5a and 5b). Thus, although the *RNS1* promoter used in these studies is sufficient to provide transcriptional control in response to both stimuli, other regulatory mechanisms seem to contribute to the different levels of induction of the *RNS1* transcript from the native gene.

To examine the possibility of post-transcriptional regulation by wounding and ABA, we designed constructs containing either the *RNS1* cDNA or genomic DNA under the control of the nopaline synthase (nos) promoter (Figures 3d and 3e). Specifically, we fused the mature *RNS1* transcribed region (RNS1cDNA) or a genomic clone corresponding to the coding region plus 5' and 3' UTR and intron sequences of *RNS1* (pre-RNS1) to the nos promoter (designated nos-RNS1cDNA and nos-preRNS1, respectively; Figures 3d and 3e). As a control for this set of constructs, we used the human β-globin gene under the control of the *nos* promoter (nos-globin, Figure 3c). These constructs contain a 'tag' of 42 nucleotides transcribed from the *nos* promoter. Thus, blots were probed with an oligonucleotide complementary to the *nos* tag to distinguish between *RNS1* transcribed from the transgene and the endogenous *RNS1* copy.

Analysis of the nos-RNS1cDNA lines revealed no difference between *nos* signal in wounded and unwounded leaves (Figure 6a and 6c), although endogenous *RNS1* was induced by wounding (not shown). In the nos-preRNS1 plants, however, a reproducible increase in *nos* signal was seen (Figure 6a and 6c). The two bands detected in the preRNS1 and RNS1cDNA lanes could be the

result of alternative polyadenylation sites, as these constructs contain both the endogenous *RNS1* and *E9* 3' end polyadenylation signals. This was confirmed by 3'RACE analysis, which identified two transcript ends corresponding to the two alternative polyadenylation sites (data not shown). Control nos-globin lines indicate that wounding does not induce the *nos* promoter (in fact, a slight but reproducible repressive effect was seen). It is therefore possible that some level of post-transcriptional regulation of *RNS1* mRNA exists that requires either the entire UTR regions or intron sequences or both, whereas the cDNA sequence alone is not sufficient. This increase of approximately 2.5-fold (Figure 6c) might provide a second layer of induction in addition to the transcriptional effect described above.

Post-transcriptional regulation was not observed after treatment with ABA (Figure 6b and 6d). Transcript levels in ABA-treated nos-preRNS1 and nos-RNS1cDNA plants resemble those in untreated plants. This disparity in post-transcriptional regulation might explain why endogenous *RNS1* is induced to higher levels by wounding than by ABA.

Tissue specific, developmental and stress regulated activity of the *RNS1* promoter

Analysis of specific patterns of expression can also provide clues to dissect the mechanisms that control *RNS1* expression. Analysis of *RNS1* promoter activity could also be used to identify transcription factors with similar expression patterns that may participate in this control. To analyze promoter activity, we used plants expressing the RNS1p-GUS construct described in figure 3. Plants expressing a similar construct in which the GUS reporter was replaced by luciferase (RNS1p-LUC) were also made.

Plants at different stages, from germination to maturity, were subjected to GUS staining (Figure 7 a-f). In the absence of stress the *RNS1* promoter is active early during germination (Figure 7 a-c). GUS staining was detected in cotyledons as early as one day after germination (Figure 7a), and almost disappeared 3-4 days after germination. Seven day-old seedlings showed expression in root tips (Figure 7c) and hydathodes (Figure 7b), and some expression could be observed in vascular tissue (Figure 7b). In adult leaves, *RNS1* expression was limited to

hydathodes (Figure 7d). In flowers, *RNS1* expression was only observed in anthers (Figure 7 e-f).

Activity of *RNS1* promoter in response to wounding and ABA was analyzed using RNS1p-LUC plants. As described previously, the *RNS1* promoter responds to wounding and ABA stimuli. Luciferase expression was observed throughout ABA-treated plants (Supplemental figure S4c), and in local and systemic tissues in wounded plants (Supplemental figure S4d). Note that *RNS1* promoter activity is higher next to the wound (the wounds can be observed in the visible light picture, Supplemental figure S4b). We were unable to detect significant luciferase activity upon dehydration of plants expressing RNS1p-LUC. However, wounding combined with dehydration produced a stronger luciferase signal than did wounding alone (data not shown).

Discussion

The regulation of *RNS1* transcript accumulation defines a novel pathway for the wounding response in Arabidopsis. The induction of RNS1 activity is independent of the two signals that have been proposed to control wounding responses in this plant – JA and OGAs. This pathway is also independent of other defense response regulators like ethylene (Reymond et al. 2000) and salicylic acid (SA; LeBrasseur et al. 2002). In this report we examined whether ABA controls this novel pathway. We showed that treatment of plants with ABA or wounding induces the expression of *RNS1* within the same timeframe. Accumulating evidence points to ABA as a component of the wounding response in plants. Although the exact nature of its contribution has not been defined, it is known that ABA accumulates upon wounding (Peña-Cortés et al. 1991). Our results with ABA mutants indicate that ABA is necessary for full induction of *RNS1* during the wounding response. Thus ABA role during wounding seems to regulate the amplitude of the wounding response for *RNS1* and likely other genes that could be co-regulated by the same pathway.

ABA is also likely to mediate the induction of dehydration-responsive genes that occurs locally following wounding. The cDNA microarray analysis carried out by Reymond et al. (2000) suggests that dehydration may also directly control wound gene induction, at least in Arabidopsis. Many of the wound-

induced genes identified in that study were also induced by dehydration. It is likely that the extent of tissue damage incurred by the plant will determine the extent to which dehydration and ABA influence gene expression during a wound response (de Bruxelles and Roberts 2002). An alternative view was presented by Cheong et al. (2002), who suggested that drought and cold response pathways are activated in response to wounding. Their hypothesis is based on microarray experiments showing that the transcription factor DREB1B/CBF and several of its downstream targets are induced after wounding.

The use of mutants that either do not produce or cannot respond to ABA allowed us to show that ABA is one part of a signaling cascade that mounts a comprehensive wound response. ABA mutants consistently showed a weaker induction of *RNS1* expression, indicating that ABA is necessary for full *RNS1* induction. However, this hormone is not absolutely necessary for *RNS1* wound induction, as 40-50% of the increase still occurs in the absence of ABA signaling (Figure 2b). These results indicate the existence of an ABA-independent pathway that is responsible for a substantial portion of the induction of RNS1 and nuclease activities after wounding. Based on our and others' previous results (Reymond et al. 2000; LeBrasseur et al. 2002), this pathway is also non-responsive to JA, OGAs, SA or ethylene. The existence of this pathway led to the previous assertion that wounding control of *RNS1* was independent of ABA (LeBrasseur et al. 2002). However it is now clear that intact ABA production and ABA signaling pathways are necessary for full induction of *RNS1*, as indicated by the results obtained with *aba1-1,* and *abi1* and *abi2* respectively.

Our experiments indicate that there is a synergy between different signals contributing to the induction of *RNS1* expression. Although *RNS1* is not significantly induced by dehydration, there seems to be a stronger induction by wounding when plants are dehydrated. It is possible that the putative DRE is not functional, or alternatively, this element present in the *RNS1* promoter may function only in a cooperative manner with other elements in the promoter. Synergistic interactions have been described before. For example, the stress-responsive gene *RD29A* is rapidly induced after dehydration by an ABA-independent pathway, which is followed by a strong ABA-dependent induction. This regulation was explained by the existence of separate *cis*-acting elements in

the *RD29A* promoter, including DRE and ABRE elements (Yamaguchi-Shinozaki and Shinozaki 1993; 1994).

We found that *RNS1* induction by wounding and ABA is controlled, at least in part, at the transcriptional level. The best characterized transcription factors that participate in ABA regulation are ABI3, ABI4 and ABI5. Although their activity has been mostly studied during seed development, it is clear that these transcription factors can also act in vegetative tissues (Arenas-Huertero et al. 2000; Rohde et al. 2000; Brocard et al. 2002). However, our results and those of Suzuki et al. (2003) indicate that ABI3, ABI4 and ABI5 are not responsible for transcriptional control during wounding induction of *RNS1*. Recently other transcription factors with the ability to bind ABREs have been described (see review by Yamaguchi-Shinozaki and Shinozaki 2005). Analysis of *RNS1* expression patterns can provide clues to identify transcription factors that could regulate its expression. Comparison of *RNS1* promoter activity with known expression patterns of other ABRE binding proteins shows a striking similarity between *RNS1* and *AREB1* expression (Fujita et al. 2005). AREB1 is a basic domain/ leucine zipper factor that binds ABREs and functions as a *trans*-activator to regulate ABRE-dependent ABA signaling that enhances drought tolerance in vegetative tissues (Fujita et al. 2005). As *RNS1*, *AREB1* is expressed in roots, hydathodes and anthers (Fujita et al. 2005); thus, it is possible that AREB1 also participates in the control of *RNS1* expression. In addition, the MYB transcription factor PHR1 has been shown to regulate *RNS1* expression in response to phosphate-starvation conditions (Rubio et al. 2001).

The *RNS1* promoter alone is able to provide some wound and ABA responsiveness to reporter genes. The *RNS1* promoter has a modular structure similar to that of other ABA-responsive genes, suggesting that similar synergistic interactions control *RNS1* expression. The promoter contains ABA-responsive elements, such as ABRE, MYB and MYC regions, and an ABA-independent, dehydration-responsive DRE element, which might mediate a quick response to dehydration even before the peak of ABA production is reached (Yamaguchi-Shinozaki and Shinozaki 1993; 1994). The binding of DREB1B to this element after its wound induction (Cheong et al. 2002) might provide a direct link between wounding and dehydration responses. These promoter elements could also

cooperate with several wounding-responsive elements in the *RNS1* promoter to produce the full induction of *RNS1* after mechanical damage, as shown for other genes (Narusaka et al. 2004). In addition to this synergy, ABREs might be promiscuous; signals other than ABA might activate ABRE-mediated transcription (reviewed by Nambara and Marion-Poll 2003). It is thus possible that ABREs act as nodes in signaling crosstalk. This possibility is supported by recent work showing that ABREs are over-represented in the promoter regions of genes corresponding to several different stress cDNA collections (Mahalingam et al. 2003). However, the presence of multiple ABRE elements in a promoter is not a random event, since only 137 genes out of 26,207 genes in the Arabidopsis genome have multiple ABREs (Huang and Wu 2006). Interactions between ABA and JA signaling during the wound response might be mediated by AtMYC2 (review by Lorenzo and Solano 2005). This hypothesis would explain microarray results that show overlaps between wounding responses and those observed after pathogen attacks, abiotic stress, and hormonal treatments (Reymond et al. 2000; Cheong et al. 2002).

The transcriptional responsiveness of the reporter constructs shown in Figure 3 and Figure 7 supports the functionality of the ABA- and wounding-responsive elements in the *RNS1* promoter. However, transcriptional activity of the promoter is insufficient to explain the differences in transcript accumulation of the endogenous *RNS1* after wounding and ABA treatment (Figure 5). Analysis of the expression of the *RNS1* transcript, including UTRs and introns and driven by a constitutive promoter, showed that untranslated sequences also respond to wounding (Figure 6a and 6b). The simplest explanation of this result is that sequences in the *RNS1* mRNA stabilize the transcript in response to wounding. In plants, several stress and hormonal responses affect mRNA stability (reviewed by Gutiérrez et al. 1999). Our results might be the first evidence of post-transcriptional control during the wounding response since, to our knowledge, changes in mRNA stability in response to this stress have not been described before.

A transcription regulatory element in the transcribed region, however, cannot be ruled out. For example, a transcriptional enhancer could be located in the *RNS1* transcribed region. Most studies on transcriptional regulation and

promoter elements that control ABA and wounding responses have focused on the promoter region upstream of the transcription start site (see, for example, reviews by Farmer et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2005).

Several recently identified ABA-hypersensitive mutants, such as *abh1*, have mutations in RNA-binding proteins. A double-stranded RNA-binding protein, HYL1 (Lu and Fedoroff 2000), and an Sm-like snRNP protein, SAD1 (Xiong et al. 2001), control ABA regulation of seed germination. Plants carrying homozygous mutations in either of these genes are hypersensitive to ABA, suggesting that both proteins are negative regulators of ABA signaling. Another RNA-binding protein, AKIP1, was identified as a specific target of the ABA-activated protein kinase AAPK (Li et al. 2002). These results prompted the idea that ABA signaling is linked to RNA metabolism (reviewed by Fedoroff 2002).

The induction of the RNS1 RNase by ABA is further support for ABA-regulation of RNA metabolism. Interestingly, although *RNS1* is induced by ABA, its expression is downregulated in the *abh1* mutant. Based on this finding, Hugouvieux et al. (2001) proposed that *RNS1* and other downregulated transcripts could be negative regulators of ABA signaling. Following this hypothesis, RNS1 would be induced by ABA early during the wounding response, and work in a negative feedback loop to regulate such response. In addition, in view of the reduced level of *RNS1* in the *abh1* mutant, it was suggested that *RNS1* itself is a target for post-transcriptional regulation by ABA (Hugouvieux et al. 2001). We were unable to detect regulation of the *RNS1* cDNA or pre-RNA by ABA. However, our experimental set-up could have interfered with this regulation. ABH1 is a cap-binding protein; therefore interactions with the 5' UTR of target transcripts could be expected. Our transgene transcripts carry a 5' nos tag to differentiate them from endogenous *RNS1*. This tag could disrupt interaction between ABH1 or an associated factor with the 5' UTR of *RNS1*. Thus, more experiments will be necessary before we can discard a role of post-transcriptional regulation of *RNS1* by ABA.

Our initial results and the tools developed in this work open a new avenue to the study of post-transcriptional regulation during wounding, an area mostly overlooked so far. It also provides a means to test directly the commonly accepted idea that ABA regulation has a large post-transcriptional component. In

addition to helping us to dissect the complex signaling pathways leading to RNS1 induction, our work has begun to address the role of ABA in the regulation of wounding response and the function of RNS1 as part of the signal or response to wounding and ABA.

Supplemental Data

Supplementary material (Figures S1, S2, S3, and S4) is available at the end of this chapter.

Acknowledgments

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Figures

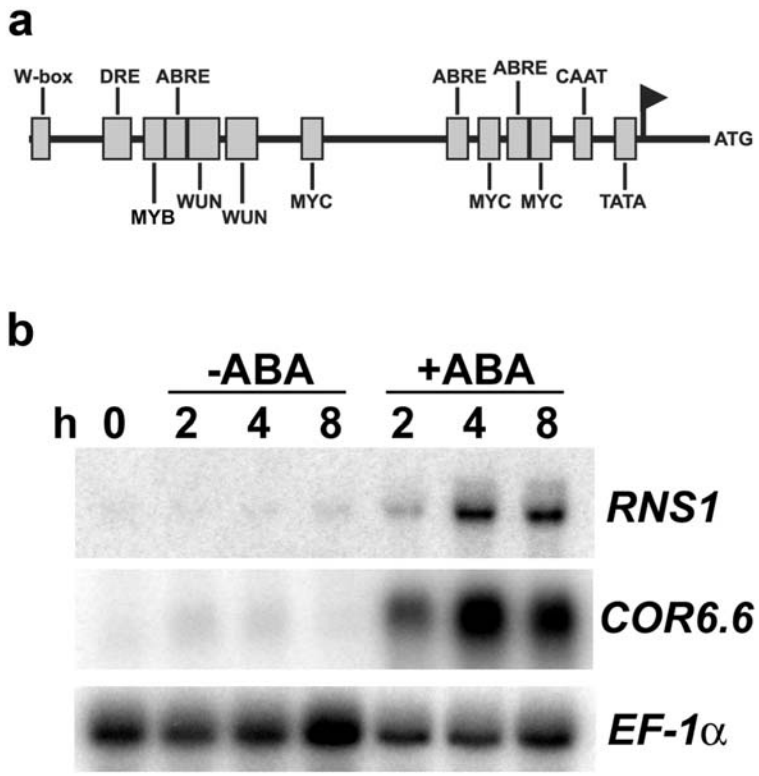


Figure 1: ABA induces *RNS1* expression. **(a)** Structure of the *RNS1* promoter. Motifs with significant similarity to previously identified *cis*-acting elements are shown (grey boxes). These include CAAT and TATA boxes, wound-responsive elements (W-box, WUN), a dehydration-responsive element (DRE), ABA-responsive elements (ABRE), and MYB and MYC binding sites. **(b)** Northern analysis of RNA isolated from seedlings treated with 100 μM ABA for the indicated times (hours). The *COR6.6* probe was used as a control for the ABA treatment, and *EF-1α* as a control for loading.

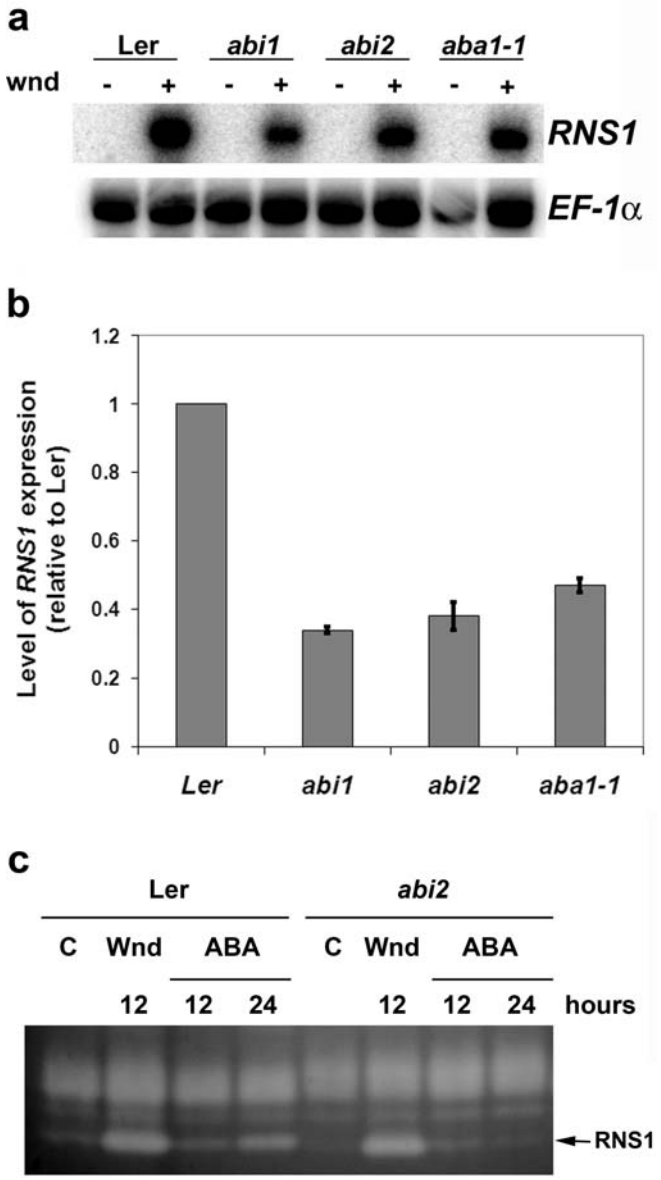


Figure 2: Participation of ABA in the wound signaling pathway that controls *RNS1* expression. Wild type (*Ler*) and mutants in ABA signaling (*abi1*, *abi2*) and biosynthesis (*aba1-1*) were examined for induction of *RNS1* after wounding. **(a)** Northern blot analysis of RNA extracted from seedlings 4 h after wounding. **(b)** Average values of the quantification of the results obtained in three experiments as the ones described in (a). Three independent experiments were performed; for each experiment individual bands were quantified and normalized using EF-1α as loading control, these values from the three experiments were then averaged and standard error was calculated. The average and error are shown in the figure. **(c)** Increase in RNS1 activity in response to ABA and wounding is compromised in the *abi2* mutant. Wild type (Ler) and *abi2* 2-week-old seedlings were examined for RNase activities. Plants were wounded for 12 h, treated for 12 and 24 h with ABA or left untreated as control. Twenty micrograms of proteins were loaded in each lane.

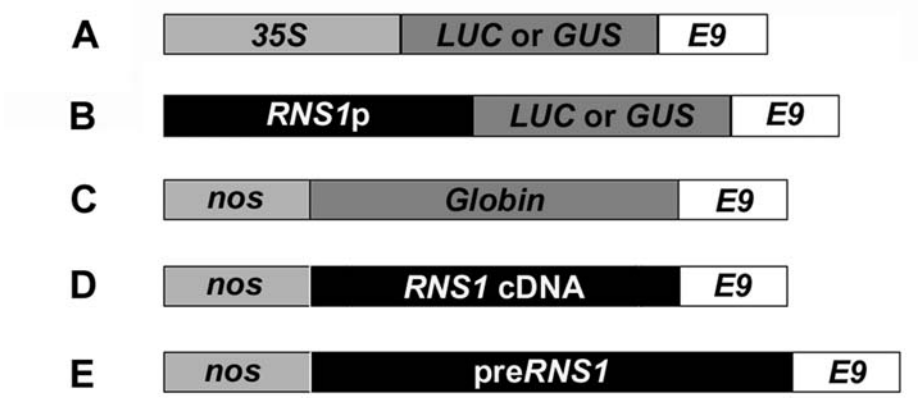


Figure 3: Constructs used to examine the regulation of *RNS1*. Several constructs were used to transform wild-type Arabidopsis plants. Transgenic lines were then used to analyze the expression of the reporters under various conditions. *LUC*, *Luciferase* coding region*; GUS*, *β-glucuronidase* coding region; *35S*, CaMV *35S* promoter; *nos*, nopaline synthase promoter; *RNS1*p, *RNS1* promoter; *E9*, 3' end of the pea *E9* gene; pre*RNS1*, transcribed region of *RNS1* including UTRs and introns.

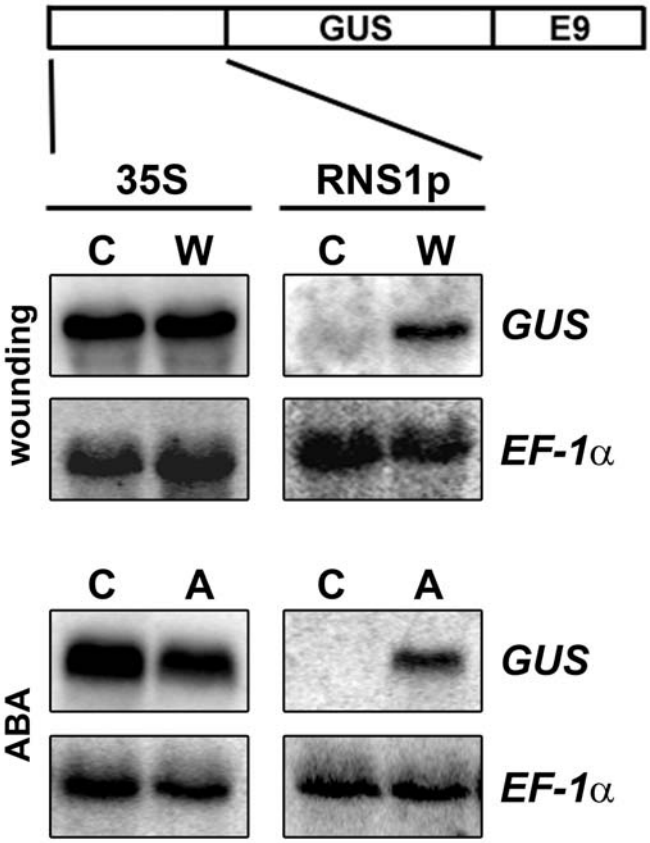


Figure 4: The *RNS1* promoter confers wound- and ABA-inducibility to reporter transcripts. Leaves of transgenic Arabidopsis plants expressing the *GUS* reporter under the control of either 2.6 kb of genomic sequence upstream of the *RNS1* transcription start site or the constitutive 35S promoter were harvested 4 h after wounding or ABA treatment (W and A respectively). Untreated plants were used a control (C) for wounding and buffer treated plants (C) were used as control for ABA treatments. Blots were probed with *GUS*, then stripped and probed with *EF-1α* (to control for loading). 35S-GUS plants were used as controls to demonstrate that *GUS* is not stabilized by wounding. For each experiment, at least 3 independently transformed lines were used. Representative results are shown.

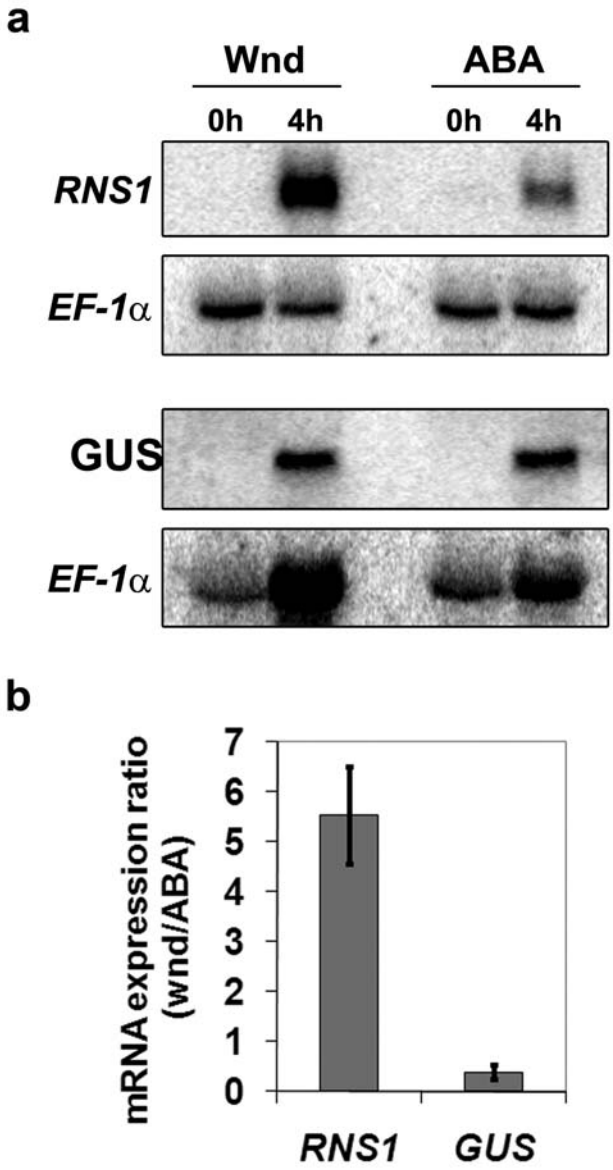


Figure 5: Differential accumulation in response to wounding and ABA of endogenous or reporter genes under the control of the *RNS1* promoter. **(a)** Northern blot analysis of transcript accumulation corresponding to the endogenous *RNS1* (upper panels) or the *GUS* reporter under the control of the *RNS1* promoter (lower panels). Blots were treated as in Figure 4. **(b)** Quantification of data shown in (a). Data represent the average normalized ratio from at least four independent experiments involving 8 independent transgenic plant lines. For each blot, the normalized values were calculated by dividing the *GUS* (or the *RNS1*) transcript level in response to wounding and to ABA to that of the *EF-1α* transcript. Only the +wounding or +ABA transcript level (each divided by that of *EF-1α*) was used to calculate the normalized ratio for a given experiment.

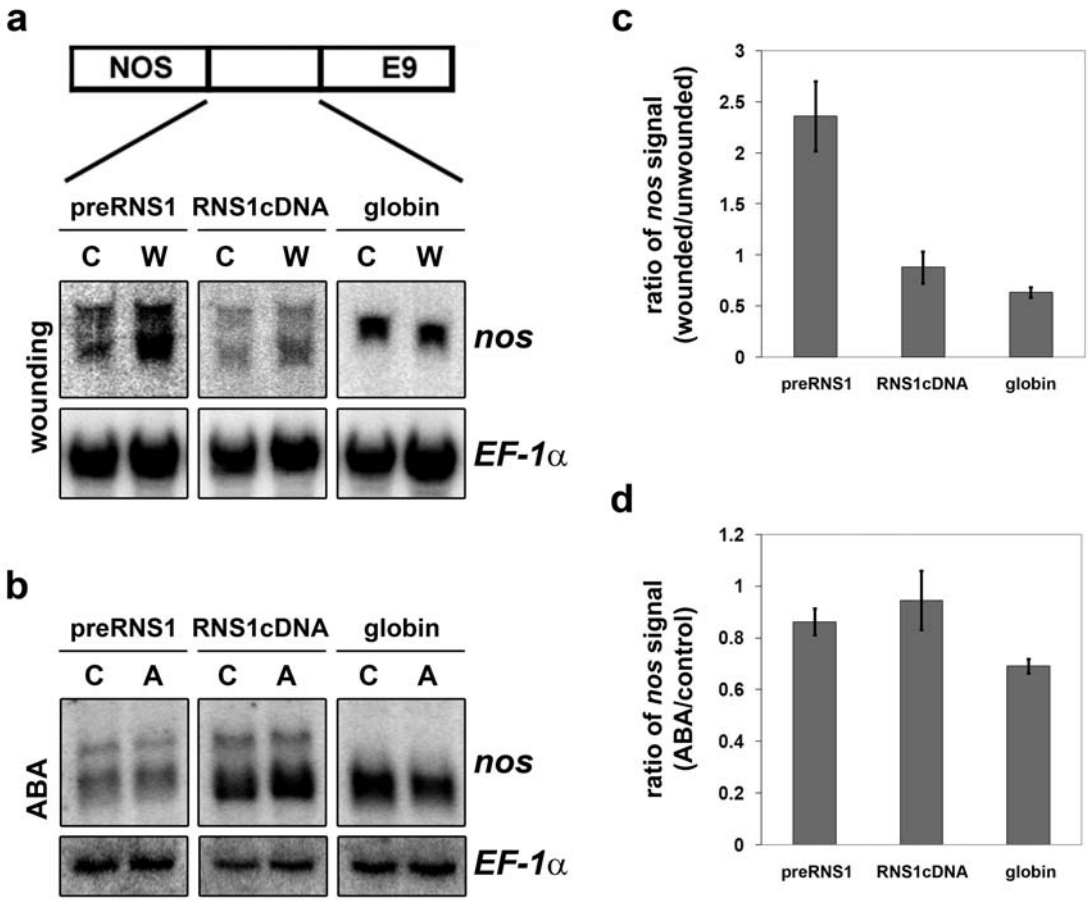


Figure 6: Differential response of *RNS1* transcribed sequences to wounding and ABA. **(a)** Pools of T_2 Arabidopsis seedlings expressing either the entire *RNS1* transcribed region (left panels), the *RNS1* cDNA (center), or the *globin* transcript (right) under the control of the constitutive *nos* promoter were wounded and harvested 3 h later. An oligonucleotide corresponding to a transcribed portion of *nos* was used as a probe in order to distinguish the transgene from endogenous *RNS1*. Blots were then stripped and probed with *RNS1* and *EF-1α* (to control for loading). **(b)** Same as (a) except that the plants were treated with 100 μM ABA and harvested 4 h later. **(c)** Average values of the quantification (see figure 2) of the results obtained in three experiments as the ones described in (a). *Nos* signal was corrected for loading differences (NOS/EF-1*α*); and it is shown as ratio of wounded vs. unwounded expression [(NOS/EF-1*α*) wounded/ (NOS/EF-1*α*) unwounded]. **(d)**. Average values of the quantification of the results obtained in three experiments as the ones described in (b). *Nos* signal is shown as ratio of ABA-treated vs. buffer-treated expression. For each experiment, at least 3 independently transformed lines were used. Representative results are shown.

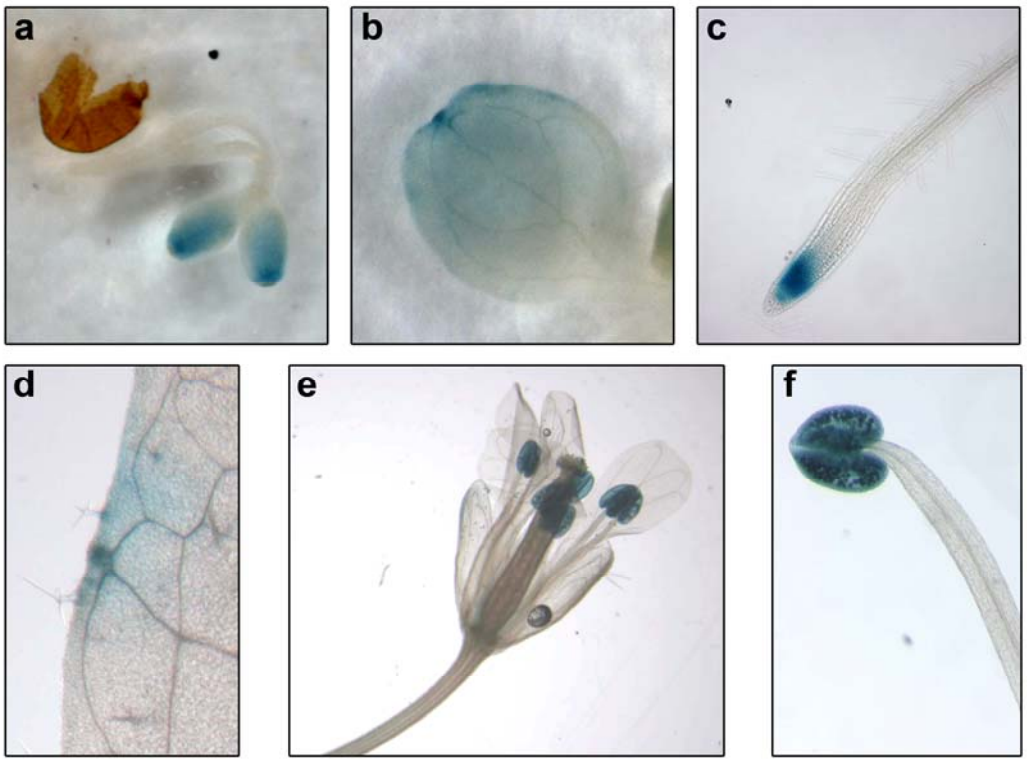
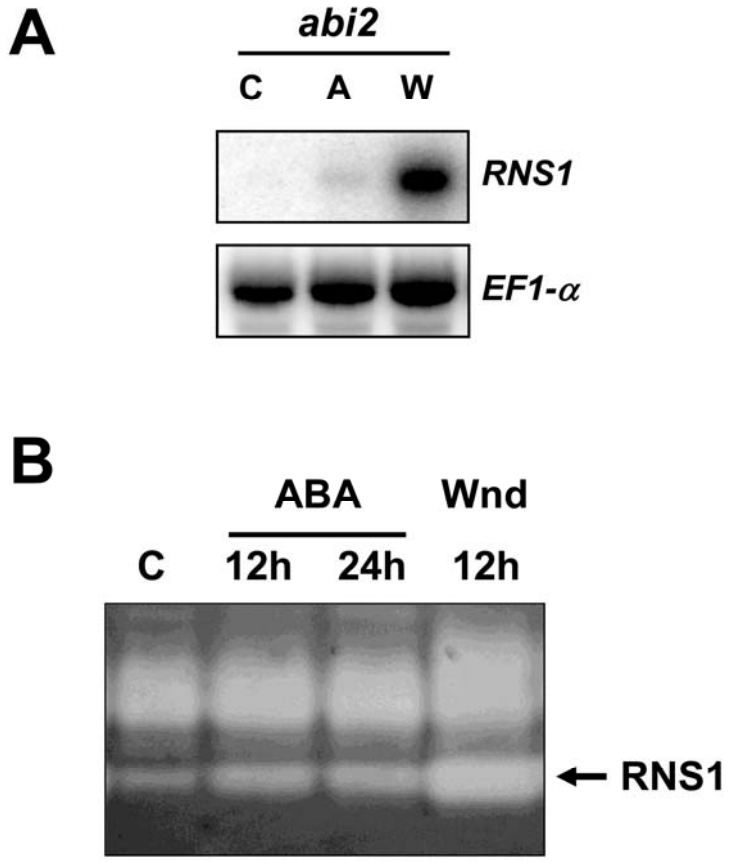


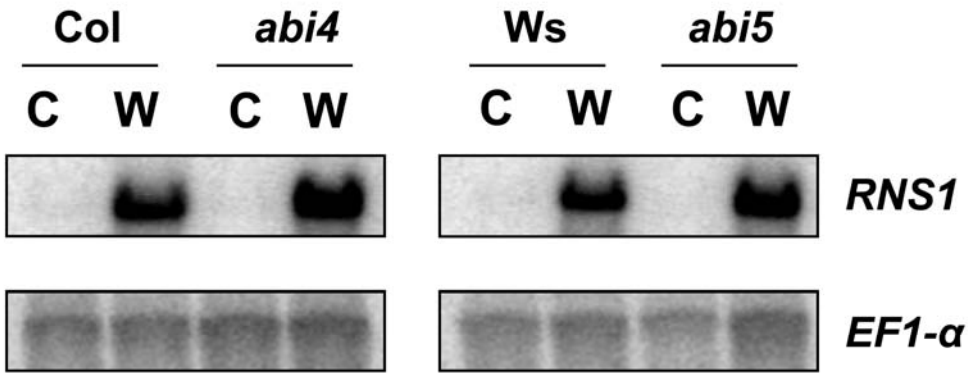
Figure 7: Tissue specific, developmental and stress regulated activity of the *RNS1* promoter. **(a-f)** Patterns of *RNS1* promoter-driven GUS expression in seedlings at different ages or in different tissues: (a) 1-d-old seedling, (b-c) cotyledon and root of 7-d-old seedling respectively, (d) 4-week-old leaf, (e-f) mature flower.

Supplemental

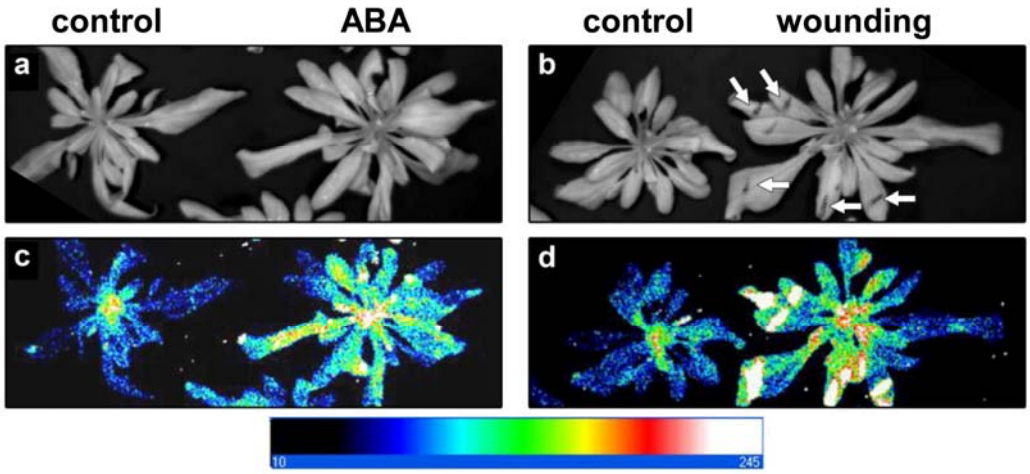
Supplemental Figure S1: Structure of the *RNS1* promoter. One kb of genomic sequence upstream of the start codon (box) of *RNS1* was analyzed to identify the presence of motifs with significant similarity to previously identified wounding- and ABA-related cis-acting elements (W-box, WUN), a dehydration-responsive element (DRE), ABA-responsive elements (ABRE), and MYB and MYC binding sites.



Supplemental Figure S2: (A) Induction of *RNS1* by ABA treatment is dependent of *abi2* signaling. Northern blot analysis of RNA extracted from *abi2* seedlings 4 hours after wounding (W) or after treatment with 100μM ABA for 4 h (A) or water (C). (B) Increase in RNS1 activity in response to ABA. Plants were treated with 100μM ABA or wounded for the indicated times. Protein extracts were prepared from these plants and 90 μg of each sample were analyzed. RNase activity was detected by an in gel activity assay. The band corresponding to RNS2 was determined by comparison with an extract from plants wounded for 12 h. Wounding samples are included as positive controls for the induction of RNS1 mRNA and activity.



Supplemental Figure S3: Induction of *RNS1* by wounding is not regulated by ABI4 and ABI5. Northern blot analysis of RNA extracted from WT (Col or Ws) and *abi4* and *abi5* mutant seedlings 4 h after wounding. Individual bands were quantified and normalized using *EF-1α* as loading control.



Supplemental Figure S4: Patterns of *RNS1* promoter-driven luciferase expression in response to wounding and ABA. Four-week-old plants were treated with 100 µM ABA (a, c, right) or wounded (b, d, right). Control plants were mock-treated (a, c, left) or not treated (b, d, left). Six hours after treatment, plants were imaged using a CCD camera without illumination to register luciferase activity (c, d). White light images are also shown (a, b). Arrows in (b) indicate wounding sites. The bar below panels c-d indicates the arbitrary scale of luciferase activity measured in those panels.

CHAPTER 3: Petunia nectar proteins have ribonuclease activity

Modified from a paper submitted to Journal of Experimental Botany

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Authors' contributions

MSH carried out the microscopy of the nectaries, the anti-microbial growth curves, RNase activity characterization of the nectar, floral organs, and stamens, gene cloning and sequencing, gene expression analyses, and motif identification. XL collected nectar, assisted with flower dissections and protein preparation, and preformed preliminary gene cloning. GL dissected petunia nectaries and isolated RNA from them for this study. RWT carried out the H_2O_2 assay. RWT and GCM conceived of the study. MSH and GCM participated in the design of the study. MSH, GCM, and RWT drafted the manuscript. All authors read and approved the final manuscript.

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Abstract

Plants requiring an insect pollinator often produce nectar as a reward for the pollinator's visitations. This rich secretion needs mechanisms to inhibit microbial growth. In tobacco nectar, anti-microbial activity is due to the production of hydrogen peroxide. In a close relative, *Petunia hybrida*, we found limited production of hydrogen peroxide; yet Petunia nectar still has anti-bacterial properties, suggesting a different mechanism may exist for this inhibition. We compared the nectar proteins of Petunia plants with those of ornamental tobacco and found significant differences in protein profiles and function between these two closely related species. Among those proteins, we identified RNase activities unique to Petunia nectar. We also cloned the genes corresponding to four RNase T2 proteins from *Petunia hybrida* that show unique expression patterns in different plant tissues. Two of these enzymes, RNase Phy3 and RNase Phy4, are unique among the T2 family and contain characteristics similar to both S- and S-like RNases. Analysis of amino acid patterns suggest that these proteins are an intermediate between S- and S-like RNases, and support the hypothesis that S-RNases evolved from defense RNases expressed in floral parts. This is the first report of RNase activities in nectar.

Keywords: nectar, Ribonuclease, Petunia, nectary, RNase T2, nectarin

Accession numbers: GQ465920, GQ465919, GQ465918, and GQ465917

Introduction

In many angiosperms, male and female sexual organs are physically located in different places on the flower or on different flowers entirely and it is difficult to transfer the male gametes (pollen, located on the anthers) to the female gametes (ova, located within the gynoecium at the base of the style). To circumvent this problem, many plants rely on animal pollinators to transfer pollen between flowers. Often these visiting pollinators are insects, however, birds, mammals, and even reptiles are known to function in pollen transfer among flowers. The visiting pollinators do not, however, do this for free. Instead, plants offer the visiting pollinators an incentive in return for pollen transfer. This reward consists of nectar, a rich concoction of sugars, amino acids, vitamins, lipids and proteins (Nicolson and Thornburg, 2007), that is freely offered to attract the pollinators to the flower where pollen transfer takes place. The composition of floral nectar suggests that it may be a good growth medium.

Floral nectar is produced from a novel floral organ termed the nectary that is generally located inside the flower, usually at its base. When pollinators scavenge inside the flower for nectar they inadvertently pick up pollen grains and transfer them when they change flowers. However, these visiting pollinators are also a hazard to the plant. By freely ranging between the reproductive tracts of many flowers, pollinators also transfer microorganisms between flowers.

However, infections of the flower are rare in plants. Initial observations identified an array of five nectarins (nectar proteins) that were secreted into the nectar of ornamental tobacco plants (Carter *et al.*, 1999) and led to the hypothesis that a major function of the nectary is to protect the gynoecium from microorganisms vectored to the flower by visiting pollinators (Thornburg *et al.*, 2003). Isolation and characterization of these proteins (Carter and Thornburg, 2000; Carter and Thornburg, 2004b; Carter and Thornburg, 2004c; Naqvi *et al.*, 2005), helped define a novel biochemical pathway, the nectar redox cycle (Carter and Thornburg, 2004a), that exists in soluble floral nectar of ornamental tobacco. This pathway produces high levels of hydrogen peroxide (up to 4 mM; (Carter and Thornburg, 2000)) via two

independent mechanisms. The nectar redox cycle begins with the developmental expression of a NADPH oxidase in the floral nectary (Carter *et al.*, 2007). NADPH oxidase produces superoxide at the nectary membrane surface. Subsequently, the superoxide dismutase Nectarin I (NEC1), the major nectar protein, directly converts superoxide into hydrogen peroxide (Carter and Thornburg, 2000). This accumulation of hydrogen peroxide is the main antimicrobial defense of tobacco nectar, since nectar treated with catalase becomes a good substrate for microbial growth (Carter *et al.*, 2007).

The production of a superoxide dismutase protein as a mechanism of floral defense against microbes is well established in tobacco plants (Carter *et al.*, 2007). The nectar proteins have been characterized from only a few species of plants. In leek (*Allium porrum*), two nectar proteins have been characterized. The first is a mannose-binding lectin and the second is alliinase (Peumans *et al.*, 1997). Proteins in these families have anti-herbivore and antimicrobial properties, suggesting a defensive role for the leek nectar proteins as well. Characterization of *Jacaranda mimosifolia* nectar identified a nectar lipase that also appears to participate in defense (Kram *et al.*, 2008).

Recently, nectarins have also been identified in extrafloral nectar. In *Acacia* spp. an invertase was identified in soluble extrafloral nectar that modified the hexose/sucrose ratio to benefit associated ant species (Heil *et al.*, 2005); and later, classical defense proteins such as the pathogenesis-related PR proteins were identified in the extrafloral nectar of these plants (Gonzalez-Teuber *et al.*, 2009). Further, the reproductive secretions of gymnosperms have also been examined and found to contain both carbohydrate-modifying enzymes and defense proteins (Poulis *et al.*, 2005; O'Leary *et al.*, 2007; Wagner *et al.*, 2007). These findings suggest that the defense of plant secretions is an important and ancient feature of plant biology.

While preliminary studies predicted that the presence of NEC1 in nectar may be widespread among the angiosperms (Carter and Thornburg, 2000), this has never been directly tested and the occurrence of many different defense proteins in other species suggest that perhaps there are many ways to protect nectar from

microbial invasion. This can only be addressed by examining nectar defense mechanisms from other closely related species. Therefore, we have examined the nectarins of a species (hybrid Petunia) that is related to ornamental tobacco. These studies, outlined below, indicate that the nectar of Petunia has a novel defense that is not related to that found in ornamental tobacco, but may be mediated by ribonucleases; furthermore, nectar defenses based upon H_2O_2 may not be as highly conserved as we had previously thought.

Ribonucleases (RNases) are proteins that have the ability to degrade RNA. There are many different classes of RNases, all members of families with specific substrate preferences and enzymatic properties (D'Alessio and Riordan, 1997; Mishra, 2002). Ribonucleases belonging to the RNase T2 family are among those proteins enriched in flower tissues and may also have a defensive role. These proteins are normally found in the secretory pathway and many accumulate in the extracellular space (Irie, 1999; Deshpande and Shankar, 2002). The S-like RNases, a subclass of RNase T2 enzymes found in all plant species (G. MacIntosh, unpublished), are commonly expressed in flowers. The three characterized S-like RNases from Arabidopsis, *RNS1-3*, are expressed at a higher level in flowers than in any other tissue (Taylor *et al.*, 1993; Bariola *et al.*, 1994; Bariola *et al.*, 1999), with *RNS1* being detected only in flowers in the absence of stress (Bariola *et al.*, 1994). Many other S-like RNases have been isolated from flowers, or cloned from pistil libraries, or their expression has been detected mainly in flowers in a diversity of species, including tobacco *RNase NE* (Dodds *et al.*, 1996), *Antirrhinum AhSL28*, a S-like RNase from Japanese pear styles (Norioka *et al.*, 2007) among others.

S-like RNases are proposed to function in two main physiological processes: nutrition, through the recycling of inorganic phosphate during periods of phosphate starvation or during senescence and other developmental stages involving cell-death; and defense against pathogens (Bariola and Green, 1997; Deshpande and Shankar, 2002). S-RNases are the other class of RNase T2 enzymes found in flowers. S-RNases participate in gametophytic self-incompatibility in at least three plant families (McClure *et al.*, 1989). S-RNases are secreted into style mucilage,

where they abort the growth of pollen bearing the same S-allele (Clarke and Newbigin, 1993). This cytotoxic activity and their expression in flowers lead to the hypothesis that gametophytic self-incompatibility may have evolved through the recruitment of an ancient flower ribonuclease involved in defense mechanisms against pathogens for use in defense against "invasion" by self pollen tubes (Hiscock *et al.*, 1996; Nasrallah, 2005).

Tobacco nectar has been well characterized. In addition to the identification of the defense mechanism and main protein complement of tobacco nectar, we have characterized the biochemical changes and key regulators of gene expression controlling nectaries development and nectar secretions (Horner *et al.*, 2007; Ren *et al.*, 2007a; Ren *et al.*, 2007b; Liu *et al.*, 2009). However, the conservation of these mechanisms in nectar from other related species is lacking. In a first attempt to extend the characterization of nectar to other species, we present here an analysis of nectar proteins from *Petunia hybrida*, which, like tobacco, belongs to the Solanaceae family. Petunia nectar has potent antimicrobial activity, but surprisingly does not produce large amounts of hydrogen peroxide, although Petunia and tobacco are closely related species. Instead, Petunia nectar contains many ribonuclease activities not found in tobacco. We identified novel RNase T2 enzymes expressed in nectaries with characteristics intermediate between S- and S-like RNases. These proteins could represent an intermediate step in the evolution of S-RNases, and support the hypothesis that S-RNases were recruited for self-incompatibility participation from an ancestral defense related role in flowers.

Results

Antimicrobial activity of Petunia hybrid nectar is not based on H_2O_2 production

Ornamental tobacco nectaries are bright orange (Figure 1a) due to the accumulation of β-carotene (Horner *et al.*, 2007). The increase in nectary carotenoids is concomitant with the accumulation of H_2O_2 in nectar (Carter and Thornburg, 2004a; Horner *et al.*, 2007); and we have proposed that the production of

β-carotene and ascorbic acid provides the counter-balancing antioxidants needed to protect nectary cells, and probably the rest of the gynoecium, from the highly oxidative environment caused by H_2O_2 (Horner *et al.*, 2007).

A direct comparison of the nectaries of *Petunia hybrida* to those of ornamental tobacco hybrid LxS8 (*Nicotiana langsdorffii x Nicotiana sanderae* var LxS8) showed that, in contrast to the ornamental tobacco, the mature nectaries of *Petunia hybrida* do not turn bright orange, but rather remain a dull yellow. This observation suggested that the biochemical processes occurring in tobacco and Petunia nectaries could be different, and that Petunia may use different mechanisms of defense against microorganisms. To test this idea, we collected nectar from both species, and measured their H_2O_2 content (Figure 1b). Tobacco nectar accumulates up to 4 mM H_2O_2, as previously reported (Carter and Thornburg, 2004b). On the other hand, H_2O_2 accumulation in Petunia nectar is more than 10-fold lower than in tobacco.

The nectar of ornamental tobacco effectively inhibits the growth of microorganisms (Carter *et al.*, 2007). This inhibition depends on the production of H_2O_2, and it is lost if nectar is treated with catalase. We found that *Petunia hybrida* nectar also possesses antimicrobial activity. Petunia nectar can inhibit growth of *Pseudomonas fluorescens, Salmonella typhimurium* and *Erwinia amylovora* (data not shown). *Petunia hybrida* nectar contains low levels of H_2O_2; however, it could be still enough to provide antimicrobial protection. To test whether H_2O_2 was involved in this antimicrobial effect we compared the inhibitory effect of Petunia and ornamental tobacco (LxS8) with or without prior treatment with catalase. The bacteria *Pseudomonas fluorescens strain A506* was used in this assay because it had previously been shown to be inhibited by LxS8 tobacco nectar (Carter *et al.*, 2007). Figure 2 shows that both tobacco and Petunia nectar inhibit the growth of *P. fluorescens*. However, this inhibition is significantly reduced after catalase treatment of ornamental tobacco nectar. On the other hand, catalase treatment had no effect on the Petunia nectar, which was still capable of inhibiting bacterial growth. This

result suggests that a H_2O_2-independent antimicrobial mechanism exists in Petunia nectar.

Petunia nectar is rich in ribonuclease activities

Because other defensive mechanisms are suggested in Petunia nectar and RNases are commonly found in flowers, we decided to look for ribonuclease activities in nectar. To determine if RNases are present in the nectar of the tobacco and Petunia plants we used an *in gel* activity assay (Yen and Green, 1991). Nectar from *Petunia hybrida* and two tobacco species (*Nicotiana tabacum* cv. Xanthi, and the ornamental tobacco hybrid LxS8) were collected and analyzed on SDS-PAGE gels in which RNA was included. After electrophoresis the gels were incubated at 3 different pHs to improve the chance of detecting any RNases present. These assays detected RNase activities in all nectar samples (Figure 3a); and in general RNases present in the nectar of all species had higher activity at an acidic pH. However, Petunia showed a more complex RNase profile. At least 8-10 bands were detected in the Petunia nectar, ranging from ~20 to 40 kDa. In contrast, only two bands were detected in LxS8 (~ 20 and 25 kDa) and additional 1-2 weak bands in *N. tabacum* cv. Xanthi in the same size range.

We also tested the different nectar samples for deoxyribonuclease (DNase) activities by *in gel* activity assay (Figure 3b). Three DNase activities were identified in Petunia nectar. Two bands (approx 30kDa and 38kDa) seem to coincide with RNase activities and show similar pH preference in DNA and RNA gels, suggesting that these two enzymes are bifunctional nucleases. Another activity of ~25 kDa seems to be a basic DNase only observed in Petunia nectar. In contrast, no DNase activity was detected in the ornamental tobacco nectar and a single activity at ~37 kDa was found in the *N. tabacum* nectar.

The differences in RNase and DNase activities between Petunia and tobacco nectars are not due to protein degradation in the samples, since the protein profiles determined by coomassie and silver staining did not show signs of proteolysis (Figure 3c). The nectarin profile of ornamental tobacco shows the major NEC1

protein at ~29 kDa and the NEC4/NEC5 doublet at ~65 kDa. NEC3 (40 kDa) and its breakdown product, NEC2 (35 kDa) are often difficult to observe. Silver staining identifies an additional band that migrates at (~10 kDa) as well as a number of minor bands that are not visible in the coomassie-stained gel. The *N. tabacum* nectar shows the NEC1 and the NEC4/NEC5 doublet and a number of minor bands. In contrast, the nectarin profile of Petunia is clearly different than that found in either of the two tobacco species analyzed. The two major proteins migrate at ~10 kDa and 38 kDa. At least 4 minor bands at approximately 28 kDa, 32 kDa, 56 kDa and 70 kDa are also present in Petunia nectar.

To determine if the RNases present in the nectar of Petunia plants were expressed solely in the nectar or were also found in other parts of the flower as well, we assayed protein extracts from different flowers parts. Petunia and ornamental tobacco flowers were dissected into six primary organs; sepal, petal, stamens, stigma, style, and ovary (including nectaries). Protein extracts were prepared from these samples and run on RNase (Figure 4a) and DNase (Figure 4b) activity gels at pH 6.0. As shown in figure 4a, it is evident that each floral organ in the two species shows a different RNase profile. Petunia has a very complex pattern of activities in the 20-27 kDa range, and few activities larger than 27 kDa. On the other hand, ornamental tobacco flowers have a series of activities in the 27-38 kDa range not observed in Petunia, but lack many of the activities in the smaller range (Figure 4a). Many of the largest sized activities seem to coincide with DNase activities (Figure 4b). While only one DNase activity was identified in Petunia samples, up to six different bands can be seen in the various tobacco floral organs. Similarities in pattern of expression and relative intensity suggest that most of the activities detected in the 27-38 kDa range correspond to bifunctional nucleases, with the exception of an activity of ~ 33 kDa expressed only in Petunia stigmas and styles that clearly has only RNase activity.

Several of the smaller RNases that are enriched in Petunia seem to accumulate preferentially in the reproductive organs rather than in sepals and petals. Activities of ~ 18, 18.5, and 20 kDa are present only in stamens (anthers +

filaments), stigmas, styles, and ovaries; and an activity of ~ 22.5 kDa is present in all samples but is highly enriched in stamens, stigmas, and styles.

The stamens from both Petunia and ornamental tobacco flowers contained the largest number of RNase activities as well as the most abundant DNase activity. Increased expression of RNases has been observed during senescence (Taylor *et al.*, 1993; Liang *et al.*, 2002; Lers *et al.*, 2006). Thus, to determine if this increase in activities was due to senescence (dehiscence) of the anthers, proteins from anthers at various stages of flower development were prepared and analyzed on RNase activity gels (Figure 5). From our analysis it is clear that most RNases present in anthers are expressed during all stages of development and not induced during senescence, i.e. no differences were observed between anthers from stage 12a (before dehiscence) and 12b (after dehiscence). However, the 18 and 18.5 kDa doublet of activities increases during anther development, while some activities in the 30-40 kDa range are only observed in the early stages.

Novel RNase T2 genes are expressed in Petunia nectaries

Since RNase T2 enzymes are commonly found in flowers, we searched for this type of transcript in Petunia nectaries. We prepared RNA from isolated nectaries and ovaries, and used RT-PCR to amplify transcripts belonging to this family. BLASTP searches of the non-redundant protein database of NCBI identified many Petunia S-RNases, but no Petunia S-like RNases. We hypothesized that any RNase T2 enzyme in nectar would belong to the S-like RNase class, since this class has been implicated in plant defense. Primers were designed based on conserved regions of S-like RNases, determined by sequence alignment of RNaseNE (GenBank accession number AAA21135), RNaseLX (GenBank accession number P80196), and RNS1 (GenBank accession number P42813). We also searched for Petunia ESTs that could correspond to RNase T2 enzymes, and primers were designed to amplify these sequences. Primer sequences are presented in Supplemental Table 1.

Using different primer combinations we were able to amplify four distinct sequences that contained the conserved active site (CAS) cassettes that define enzymes belonging to the RNase T2 family (Irie, 1999). These were named *RNase Phy1*, *RNase Phy3*, *RNase Phy4* and *RNase Phy5,* and were deposited in the GenBank as accessions GQ465920, GQ465919, GQ465918, and GQ465917, respectively. BLASTP analysis (Figure 6) of the predicted proteins encoded by these partial sequences indicated that RNase Phy1 has 96% similarity and 90% identity to RNase NE from tobacco. Likewise, RNase Phy5 showed high homology (95% similarity, 88% identity) to tomato RNase LX. However, BLAST analyses of RNase Phy3 and RNase Phy4 resulted in hits with low sequence homology, either at the nucleotide or the amino acid levels. RNase Phy3 closest homolog was also RNase NE, but with only 33% identity and 52% similarity, and large gaps. RNase Phy4 closest homolog was an S-RNase, S42-RNase from *Pyrus x bretschneideri*, and the homology was even lower than for RNase Phy3 (29% identity, 48% similarity). In both cases homology was higher around the two CAS that define this family of enzymes. Due to their unique sequences *RNase Phy3* and *RNase Phy4* were subsequently chosen for rapid amplification of cDNA ends (RACE) analysis to determine their complete transcript sequence.

RACE PCR analysis of *RNase Phy3* yielded a partial transcript. 5' RACE was unsuccessful in yielding a complete 5' end; however sequencing analysis did reveal the first and second CAS sites. The partial *RNase Phy3* transcript is 639 nucleotides long. The predicted protein encoded by this gene has an estimated molecular weight of 23.8 kDa, and an isoelectric point of 9.25, and it is probably N-glycosylated. RACE PCR of *RNase Phy4* yielded a full length transcript of 861 nucleotides. The encoded protein showed a putative signal peptide of 19 aa. The molecular weight of the mature protein is 25.79 kDa, with an isoelectric point of 8.98. RNase Phy4 may have up to 3 possible N-glycosylation sites. RNase Phy3 has a 38% identity and a 63% similarity with RNase Phy4. BLASTP analyses (not shown) indicated that these two proteins have similar homology to S-RNases and S-like RNases, and are not clear members of either class.

Tobacco nectarins are expressed exclusively in nectaries that are actively secreting nectar (NEC1, NEC4, and NEC5; (Carter and Thornburg, 2003; Carter and Thornburg, 2004c; Naqvi *et al.*, 2005)) or in nectaries and a few other floral tissues (NEC3;(Carter and Thornburg, 2004b)). The four Petunia RNases were cloned from nectary and/or ovary cDNA. To analyze whether their expression was limited to these organs or found throughout the plant, we extracted RNA from different flower and vegetative tissues and tested for the presence of the corresponding transcripts using RT-PCR (Figure 7). Each of the four RNases was expressed in ovaries and, in addition, *RNases Phy1, 3* and *4* were also detected in nectaries. *RNase Phy1* was expressed ubiquitously throughout the plant, and although our analysis is only semi-quantitative, its expression does seem higher in floral organs than in vegetative tissues. *RNase Phy3* and *RNase Phy4* had similar expression profiles. Both were expressed exclusively in flowers, with strong expression in ovaries and nectaries. *RNase Phy4* was also highly expressed in petals and weakly detected in styles, while *RNase Phy3* was highly expressed in stigmas, but also was detected in styles and petals. *RNase Phy5* was mostly expressed in styles, although weak expression was also observed in petals, stamens (anthers), and ovaries. Thus, only *RNase Phy3* and *RNase Phy4* have patterns consistent with that of nectarins. These results suggest a role for these proteins in nectar.

RNase Phy3 and RNase Phy4 have characteristics of S- and S-like RNases

Plant members of the RNase T2 family are classified in three groups based on their phylogenetic relationships, their protein properties and their genomic organization (Igic and Kohn, 2001). Classes I and II include the S-like RNases, which are acidic enzymes with either less than four introns (Class I) or more than four introns (Class II). Class III includes S-RNases and “relic” S-RNases (Golz *et al.*, 1998). Relic S-RNases are believed to have originated from duplication of S-RNase genes but do not participate in self-incompatibility. Most S-RNases and relic S-RNases are basic proteins and have only one intron, with the exception that S-RNases of the genus *Prunus* have two introns (Yamane *et al.*, 2003). RNase Phy3

and RNase Phy4 show low homology to both S-like and S-RNases; and they have characteristics from each of these classes. These two Petunia RNases are basic proteins, as are most S-RNases; but their expression patterns do not resemble S-RNases, which are expressed mainly in the pistil. In contrast, RNase Phy3 and RNase Phy4 are also found in nectaries, ovaries and petals.

Amino acid patterns have also been used to differentiate between S-like and S-RNases. Vieira et al (2008) described four amino acid patterns that can be used to distinguish between these two classes of RNases. Two patterns were identified exclusively in S-RNases (patterns 1 and 2, yellow shade in Figure 8), and also two were used to define S-like RNases (Patterns 3 and 4, pink shade in Figure 8). In their analysis Vieira et al. identified pattern 1 in 467 of 468 S-RNases analyzed, while pattern 2 was found in 689 of 691 possible S-RNase sequences. On the other hand, the amino acid pattern (Schmid *et al.*)HEW (pattern 3) was found in 54 of 69 S-like RNases and but only in 7 of 658 S-RNase sequences (each of these 7 sequences belonged to the genus *Prunus*), and pattern 4 was found in 64 of 69 S-like RNases studied, and was not found in any of the 658 S-RNase sequences used in that study (Vieira *et al.*, 2008).

RNase Phy1 and RNase Phy5 contain the two S-like RNase patterns (Figure 8). However, RNase Phy3 and RNase Phy4 do not match either class. RNase Phy3 contains patterns 2 and 3, corresponding to S- and S-like RNases respectively (Figure 8). RNase Phy4 contains only pattern 3, indicative of S-like RNases (Figure 8), but does not have pattern 4. Thus, RNase Phy3 and RNase Phy4 show characteristics that are intermediate between S-RNases and S-like RNases, although RNase Phy4 seems to be closer to S-like RNases.

Discussion

Although the importance of nectar in pollination is well-recognized, the proteins that are present in this plant secretion, and in particular the proteins involved in antimicrobial activities are in general not well-studied. The best-studied example is the nectar from ornamental tobacco. Several nectarins, proteins present

in nectar, have been described for this plant (Carter and Thornburg, 2000; Carter and Thornburg, 2004b; Carter and Thornburg, 2004c; Naqvi *et al.*, 2005). These proteins function in the nectar redox cycle, a biochemical pathway that produces high levels of hydrogen peroxide as an antimicrobial agent (Carter *et al.*, 2007). Ornamental tobacco nectaries are bright orange due to the accumulation of β-carotene (Horner *et al.*, 2007), which, together with ascorbic acid, provides the counterbalancing antioxidants needed to protect nectary cells, and probably the rest of the gynoecium, from the oxidative environment caused by H_2O_2. The initial observation that nectaries from a closely related plant species, Petunia, did not present orange nectaries suggested that a different antimicrobial mechanism could be present in this plant. We found that *Petunia hybrida* nectar is relatively low in H_2O_2 levels and further, addition of catalase has no effect on the antibacterial activity of Petunia nectar. Thus, the strong antibacterial activity found in Petunia nectar was not dependent on the accumulation of H_2O_2.

We also found that Petunia nectar is rich in nuclease activities, in particular RNases, although DNases are also detected in this nectar. In contrast, while present, these enzymes are not detected at high levels in tobacco nectar. Differences in the patterns of RNase and DNase activities between these two plants are not limited to nectar. Other floral parts also show differential patterns, with enrichment in RNases in the 20-27 kDa range in Petunia, and enrichment in activities probably corresponding to bifunctional nucleases in the 27-38 kDa range in tobacco. Increased levels of nuclease activities, both RNases and DNases, have been observed in many plants in response to bacterial, viral and fungal pathogens (Lusso and Kuc, 1995; Floryszak-Wieczorek and Gniazdowska-Skoczek, 2001; Šindelářová and Šindelář, 2001; Kiba *et al.*, 2006), suggesting that these enzymes could have antimicrobial effects.

Nucleases are also involved in senescence and other programmed cell death processes (Dahiya, 2003). Thus, it is possible that some of the activities identified in our analysis are associated with senescence, which occurs rapidly for several floral tissues (O'Neill, 1997). This hypothesis, however, is not supported by the fact that

most activities were found in anthers, the most RNase-rich tissue in flowers, before dehiscence. Thus, it is likely that at least some of these activities are performing biological functions not related to senescence.

Analyses of gene expression have identified two families of plant RNases as part of plant defense responses, pathogenesis related PR-10 proteins (Liu and Ekramoddoullah, 2006), and S-like RNases (Bariola and Green, 1997). In this study we focused our attention on the latter. Since S-like RNases have several highly-conserved amino acid motives, we were able to amplify four Petunia S-like RNases that had not been previously described. Two of those RNases, RNase Phy1 and RNase Phy5, were highly similar to well-characterized proteins from tobacco and tomato, respectively, and their expression patterns suggested that they may not be Petunia nectarins. On the other hand, RNase Phy3 and RNase Phy4 were expressed in a pattern similar to that found for tobacco nectarins, suggesting that these enzymes may be part of the Petunia nectar defense repertoire.

S-like RNases have been implicated in defense responses against a variety of pathogens. Expression of the extracellular RNase NE from tobacco is induced by *Phytophthora parasitica* (Galiana *et al.*, 1997). Purified RNase NE inhibits hyphal growth from *P. parasitica* zoospores and from *Fusarium oxysporum* conidia *in vitro*, and co-infiltration of tobacco leaves with RNase NE and *P. parasitica* zoospores inhibited hyphal growth of the oomycete *in vivo* (Hugot *et al.*, 2002). Expression of the related *RNase NGR3* and *RNase Nk1*, from different tobacco species, is also induced in response to tobacco mosaic virus and cucumber mosaic virus respectively (Kurata *et al.*, 2002; Ohno and Ehara, 2005). In addition, Arabidopsis *RNS1* is highly induced in response to mechanical damage both in local and systemic tissues (LeBrasseur *et al.*, 2002; Hillwig *et al.*, 2008). Tobacco RNase NW, Zinnia ZRNase II and tomato RNase LE are also induced by wounding (Ye and Droste, 1996; Kariu *et al.*, 1998; Lers *et al.*, 1998). It has been suggested that the role of these secretory proteins during the wounding response is to block the spread of microorganisms that could penetrate through the wound site (LeBrasseur *et al.*, 2002).

The regulation of S-like RNases by varied pathogens and wounding suggest that these enzymes could have broad-spectrum antimicrobial activity that could be associated with cytotoxic properties of these proteins. In fact, it has been proposed that S- RNases involved in self-incompatibility likely evolved from S-like RNases that had a defensive role (Hiscock *et al.*, 1996) (Nasrallah, 2005). S-RNases have a cytotoxic effect on the pollen tube during self-incompatible pollination. It is thought that as the pollen tube elongates, the S-RNases are secreted into the extracellular matrix and may gain access into the cytoplasm of the pollen tube where they may degrade RNA from incompatible pollen (McClure and Franklin-Tong, 2006).

Secretory ribonucleases also have a defense role in animals. Several members of the vertebrate-specific RNase A family have antimicrobial properties. Human RNase 2 and RNase 3, two eosinophil associated RNases, have antiviral activity, and RNase 3 also has an antibacterial function. Angiogenin and RNase 7 have antibacterial and antifungal activities (reviewed in (Boix and Nogues, 2007)). Similarly, several zebrafish RNases, also members of the RNase A family, were shown to have antibacterial effect (Cho and Zhang, 2007). However, enzymatic activity is not essential for eosinophil associated RNases antimicrobial activity (Rosenberg, 1995; Torrent *et al.*, 2009). It has been proposed that their antimicrobial activity is due to membrane destabilizing properties of these proteins. Positively charged amino acid residues in these proteins are thought to be important to disrupt negatively charged bacterial cell membranes and may be key to their bactericidal activity ((Cho and Zhang, 2007), and references therein). Interestingly, while most S-like RNases are acidic proteins, RNase Phy3 and RNase Phy4 have high isoelectric points, indicating enrichment in basic amino acids. Thus, it is possible that the very basic nature of these proteins could indicate an antibacterial activity that can explain the effect on bacterial growth observed in our experiments.

In plants, RNase T2 proteins are divided in two classes, S-RNases and S-like RNases, based on biological role, and phylogenetic relations (Igic and Kohn, 2001). However, some proteins do not fit this classification. Relic-RNases are RNases that are no longer associated with self-incompatibility, but they are clearly derived from

S-RNases through gene duplication events (Golz *et al.*, 1998). Others, referred as non-S RNases, seem to have intermediate characteristics between S-RNases and S-like RNases (Yamane *et al.*, 2003). RNase Phy3 and RNase Phy4 seem to fall into the latter category.

Both RNase Phy3 and RNase Phy4 are basic proteins, and RNase Phy3 has only one intron interrupting the coding region (M. Hillwig and G. MacIntosh, unpublished). These are characteristics of S-RNases. However, the *RNase Phy4* gene is unusual because it does not have introns (M. Hillwig and G. MacIntosh, unpublished). In addition gene expression analyses showed that the expression pattern of RNase Phy4 (petals, ovaries and nectaries) is very different than that of S-RNases, which are mainly expressed in pistils; *RNase Phy3* is also mainly expressed in ovaries and nectaries, although in this case expression in stigma is also high. Analysis of the amino acid patterns present in both proteins also show that these proteins differ from both the canonical S- and S-like RNases, since RNase Phy3 has one of the two amino acid patterns characteristic of S-RNases, and one of the two patterns belonging to S-like RNases. RNase Phy4 only has one of the two S-like patterns, and none of the S-RNase patterns.

Yamane *et al*. (2003) identified a non-S RNase from *Prunus avium*, RNase PA1, that is also basic and has an expression pattern similar to S-RNases, but which has low level of homology with this class of proteins; in addition, phylogenetic analyses placed RNase PA1 outside of the S-RNase class. These authors proposed that this non-S RNase is a possible ancestral form of S-RNases. So far, this type of enzyme has been found only in other plants of the genus *Prunus* (Yamane *et al.*, 2003; Banovic *et al.*, 2009).

While RNase Phy3 and RNase Phy4 do not have high sequence homology to the *Prunus* non-S RNases, they do share their intermediate nature between S- and S-like RNases. Thus, these Petunia proteins could be the Solanaceae equivalent of the *Prunus* enzymes, and represent an ancestral form of S-RNases. We have some evidence that these “proto-S RNases” are conserved in tobacco and tomato (M. Hillwig and G. MacIntosh, unpublished). The potential role of these enzymes as

antimicrobial agents in nectar is consistent with the hypothesis that S-RNases were derived from enzymes involved in defense mechanisms against invading pathogens (Hiscock *et al.*, 1996; Nasrallah, 2005).

Materials and Methods

Plant Material

Petunia hybrida were obtained from a local market. *Nicotiana tabacum* cv. Xanthi was obtained from Dr. C.A. Ryan, Washington State University. The ornamental tobacco hybrid LxS8 (*Nicotiana langsdorffii* x *Nicotiana sanderae* var LxS8) was described previously (Kornaga *et al.*, 1997; Carter *et al.*, 1999). Plants were grown to floral maturity in a greenhouse with supplemental light (16 h day/ 8 h night). Nectar was collected as described in Carter *et al*. (1999) approximately 6 hours after watering to ensure adequate nectar production. For RNA and protein extraction, tissues from different floral parts were harvested at the appropriate floral stage following the classification of Koltunow et al. (1990).

FOX assay for hydrogen peroxide

Hydrogen peroxide was assayed in nectar essentially as described (Bleau *et al.*, 1998). Briefly, one ml of fresh FOX reagent (25 mM sulfuric acid, 100 µM xylenol orange, 100 µM D-sorbitol, and 250 µM ferrous ammonium sulfate) was added to 200 µl of diluted nectar. After incubating for 20 minutes at room temperature, the levels of hydrogen peroxide were quantitated spectroscopically at 560 nm and calculated using a hydrogen peroxide standard curve (up to 300 µM).

Bactericidal Assay

Raw nectar was diluted 1:1 with 10 mM phosphate buffer (pH 7.0) and a fraction was treated with catalase (SIGMA) as described in Carter et al. (2007) for 20 min. Then, 90 µl aliquots of filter-sterilized nectar were used to test bacterial growth in a 96-well microplate. *Pseudomonas fluorescens* (strain A506) was grown in LB medium overnight at 28^{o}C in the presence of 50 mg/L rifampicin. The bacterial

culture was then diluted to an OD_{600} = 0.5. Ten µl of culture were added to each microplate well containing nectar from ornamental tobacco LXS8, *Petunia hybrida*, or phosphate buffer; with or without catalase treatment. Triplicate plates were incubated in a plate reader with agitation for 18hrs at 28°C and the OD_{600} was measured every 30 min. Growth was normalized (to t=0 for each well). Each treatment was assayed a minimum of 3 times.

In vitro Gel Assay

Raw nectar was collected from *Petunia hybrida*, *Nicotiana tabacum* cv Xanthi, and ornamental tobacco plants LxS8, and stored at -80^{o}C until use. Fifty µl of nectar were analyzed on RNase and DNase activity gels as described by Yen and Green (1991). For tissue specific protein analysis a minimum of 6 flowers (stage 12) were dissected to obtain sepals, petals, stamens, stigmas, styles, and ovaries (including nectaries). Tissue was ground using mortar and pestle with liquid N_2, and extracted as described by MacIntosh et al (1996), except that the extraction buffer did not include polyvinyl polypyrrolidone and 2-mercaptoethanol. Protein concentration was determined using the Bio-Rad Protein Assay Kit, and 100 µg of total protein were analyzed in RNase or DNase activity gels. For anther/stamen analysis, at least 6 flowers at each stage (2, 6, 9, 11, 12a, 12b) were collected and stamens were harvested for protein isolation as stated above. Each activity gel is a representative of 2 independent protein isolations, and at least 3 replicates.

Protein integrity was determined by SDS-PAGE analysis. After electrophoresis, gels were stained with Coomasie Blue using GelCode Blue Stain Reagent (Pierce/ Thermo Scientific) or silver-stained according to published procedures (Blum *et al.*, 1987).

Cloning of RNases

Nectaries were isolated from *Petunia hybrida* flowers as described for ornamental tobacco (Carter *et al.*, 1999). RNA was extracted from ovaries and nectaries using the Qiagen RNeasy Plant Mini Kit, and cDNA was synthesized using

the i-Script Select Kit (Bio-Rad). To amplify cDNAs corresponding to RNase T2 homologs, we designed primers corresponding to conserved nucleotide regions by comparing sequences from Arabidopsis *RNS1* (Taylor and Green, 1991), tobacco *RNase NE* (Dodds *et al.*, 1996), and Petunia *RNase X2* (Lee *et al.*, 1992). Primers were also designed based on Petunia ESTs with homology to RNase T2 sequences. The primers used are presented in Supplemental Table 1. PCR products were cloned into pGEM T-EASY or pGEM T vector (Promega) for sequencing purposes. RNase Phy3 and RNase Phy4 were subjected to rapid amplification of cDNA ends (RACE)-PCR using GeneRacer Kit (Invitrogen). DNAs were sequenced at the Iowa State University DNA Facility. The Petunia RNase clone sequences were deposited in GenBank as accessions GQ465917 to GQ465920.

RT-PCR

Sepals, petals, stamens, stigma, styles, ovaries (with nectaries), nectaries alone, leaves, roots, and stems from *Petunia hybrida* were collected, and RNA was extracted as described above. Genomic DNA was removed using DNA-free kit (Applied Biosystems), and cDNA was synthesized using the i-Script Select Kit (Bio-Rad). PCR was performed using GoTAQ 2X Master Mix (Promega). PCR products were run on 1% TBE gels and stained with ethidium bromide. Amplification of 18S RNA was used as control for loading.

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Figures

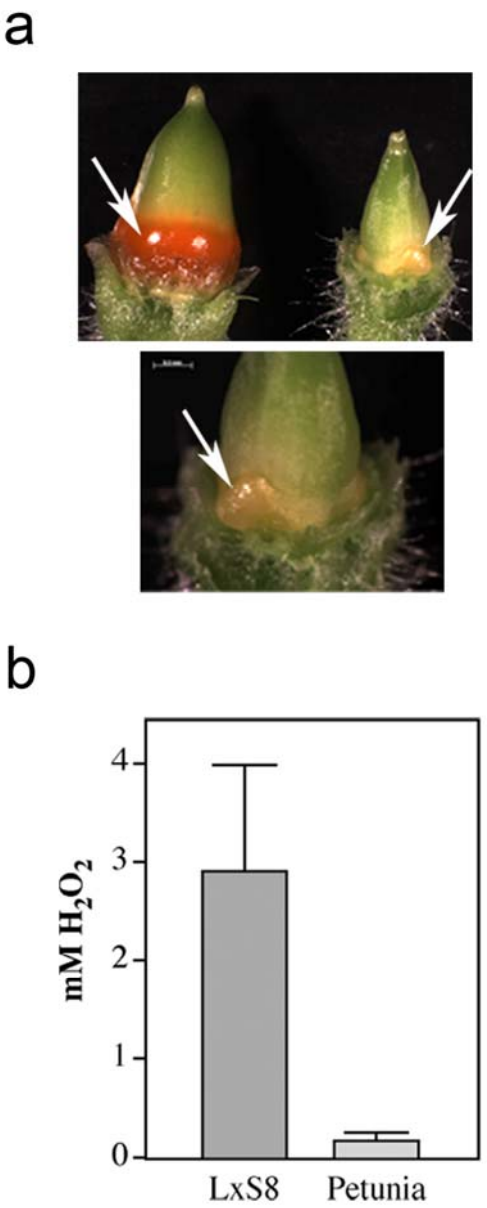

Figure 1: Differences in nectary appearance and nectar composition between Petunia and tobacco. **a.** Appearance of Petunia (right in upper panel, and lower panel) and the LxS8 tobacco hybrid (left, upper panel) nectaries (arrows) from flowers at stage 12 (Koltunow *et al.*, 1990). Observe differences in size and color; small, light yellow nectaries in Petunia, large, bright orange nectaries in tobacco. **b**. Accumulation of hydrogen peroxide in Petunia and tobacco nectar. Nectar collected from at least 20 different flowers was pooled and analyzed for H_2O_2 presence using a colorimetric assay.

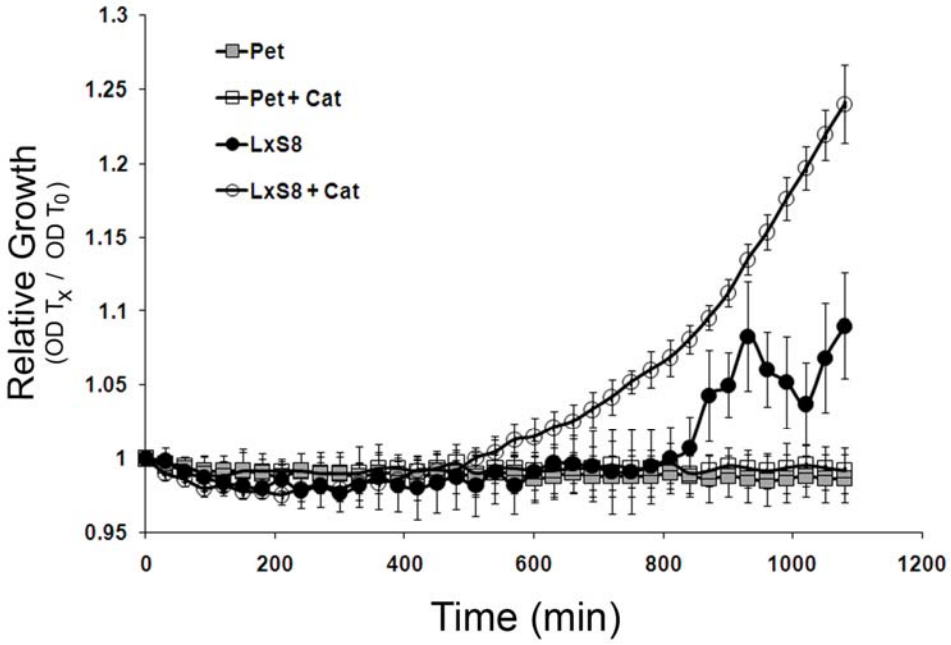

Figure 2: Effect of tobacco (circles) and Petunia (boxes) nectar on the growth of bacteria. Growth of *Pseudomonas fluorescens* (strain A506) in raw nectar (filled symbols) or nectar that was preincubated with catalase (empty symbols) was followed by changes in OD. Each point represents the mean ± SD (n=3). Data are representative of two independent experiments.

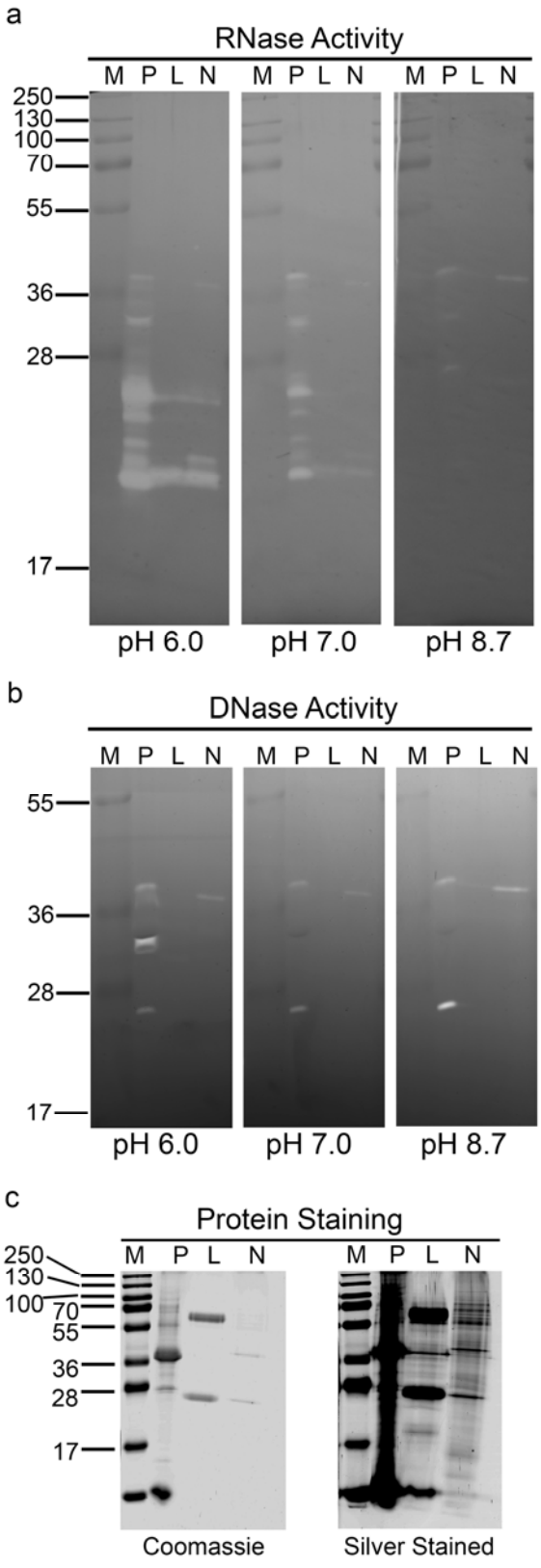
a
RNase Activity
M P L N M P L N M P L N
250
130
100
70
55
36
28
17
pH 6.0
pH 7.0
pH 8.7
b
DNase Activity
M P L N M P L N M P L N
55
36
28
17
pH 6.0
pH 7.0
pH 8.7
c
Protein Staining
M P L N M P L N
250
130
100
70
55
36
28
17
Coomassie
Silver Stained

Figure 3: Nuclease activities are present in nectar. **a**. Aliquots (50 µl) of raw nectar from *Petunia hybrida* and two different tobaccos (*Nicotiana tabacum* cv. Xanthi and the hybrid *Nicotiana langsdorffii* x *Nicotiana sanderae* var LxS8) were analyzed in an *in gel* RNase activity assay at three different pHs. P, Petunia; L, LxS8; N, Xanthi. Size (kDa) of molecular weight markers (M) is indicated. **b**. Same samples as in A, but analyzed in an *in gel* DNase activity assay. **c**. Same samples as in A, analyzed by SDS-PAGE, and stained as indicated. Gels are representative of at least 3 independent experiments.

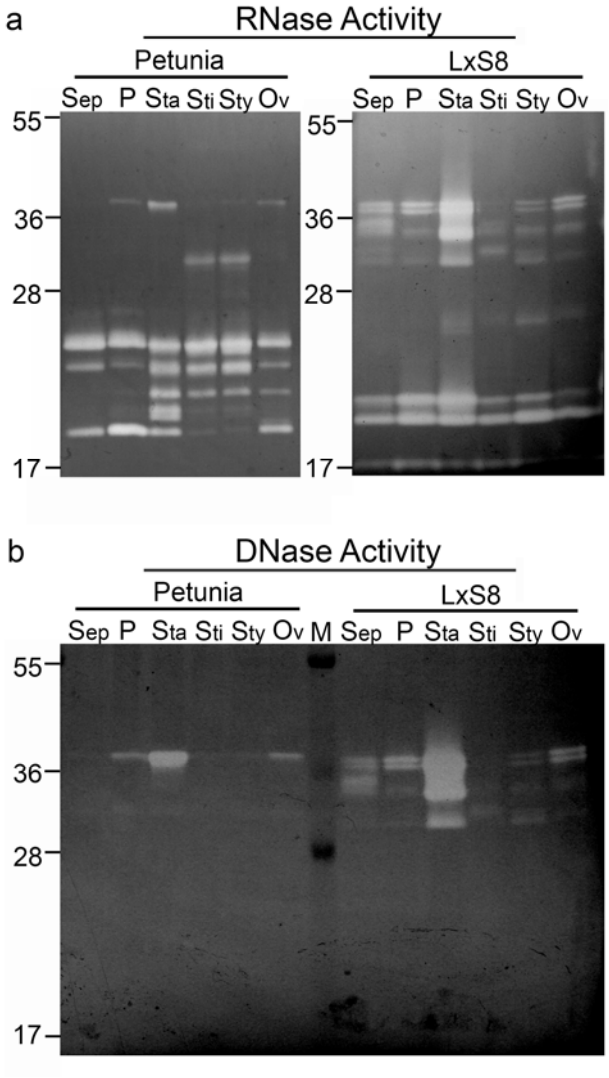

Figure 4: Nuclease profiles of different floral parts of Petunia and ornamental tobacco plants. Flowers were harvested at stage 12, and dissected to obtain sepals (Sep), petals (P), stamens (Sta), stigmas (Sti), styles (Sty) and ovaries (including nectaries, Ov). Total protein extracts (100 µg) from each floral part were analyzed in an *in gel* RNase activity assay (**a**) or DNase activity assay (**b**) at pH 6.0. Position of molecular weight markers (kDa) is indicated. Gels are representative of at least 3 independent experiments.

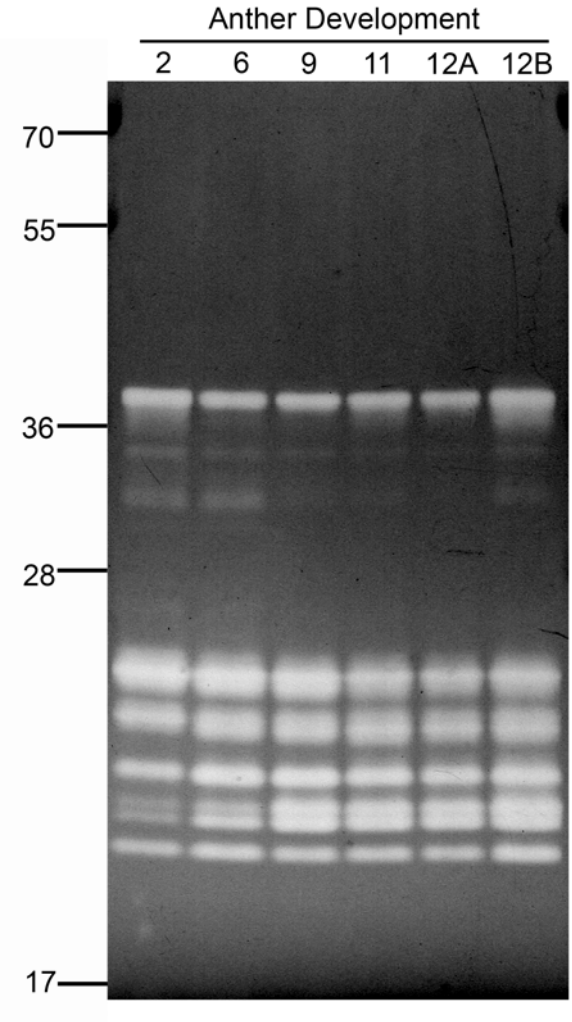

Figure 5: RNase profile of Petunia stamens during development. Stamens were collected from flowers at pre-dehiscence (2, 6, 9, 11, 12A) and post-dehiscence (12B) stages. Total protein extracts (100 µg) were analyzed in an *in gel* RNase activity assay at pH 7.0. Position of molecular weight markers (kDa) is indicated. Gel is representative of at least 3 independent experiments.

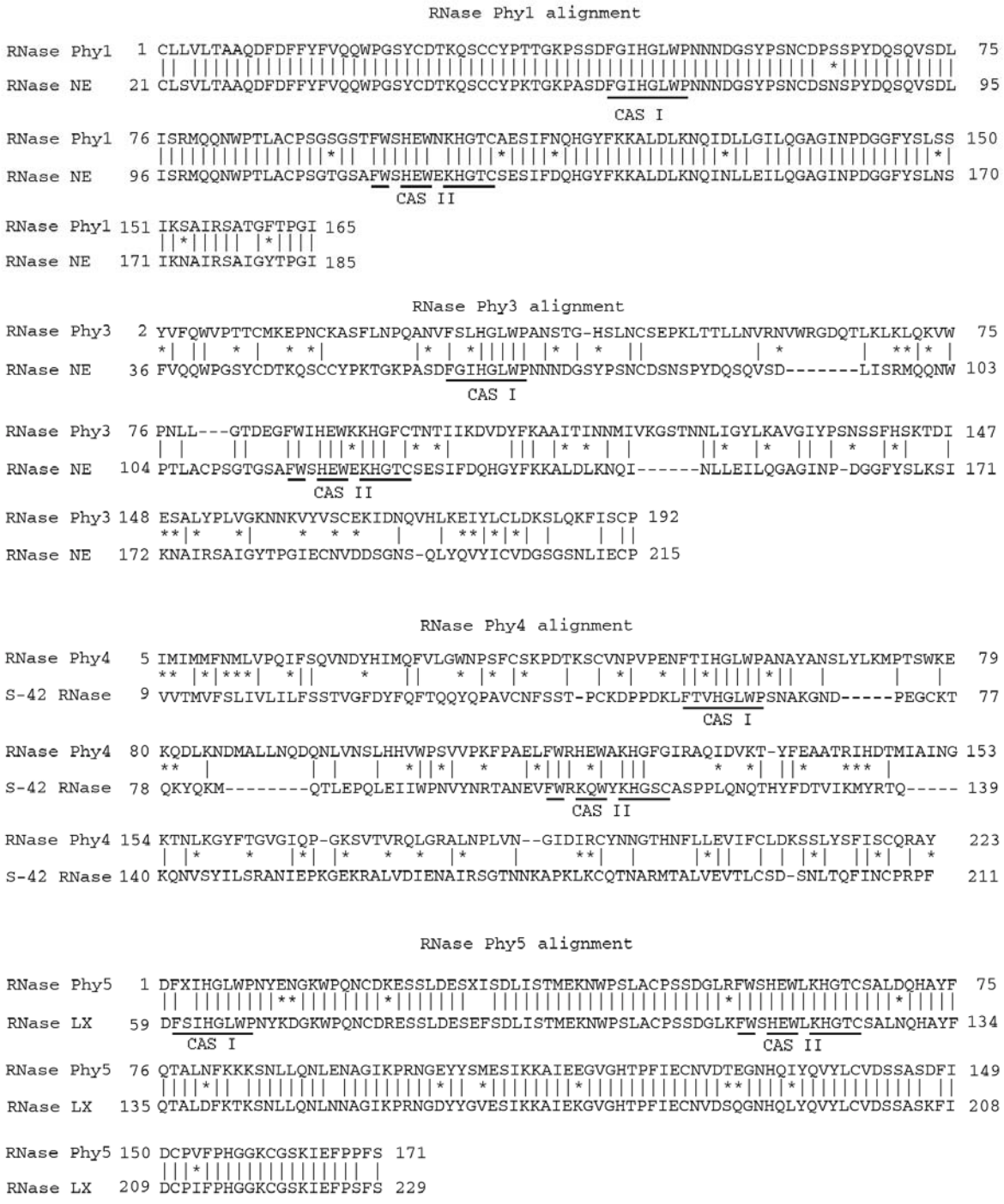

Figure 6: Petunia RNases have homology to RNase T2 enzymes from other plants. BLAST analysis of predicted RNases encoded by Petunia cDNAs amplified from ovaries and nectaries RNA. Alignment of each Petunia RNase (RNase Phy1, RNase Phy3, RNase Phy4, and RNase Phy5) with the homolog with the highest BLAST score is shown.

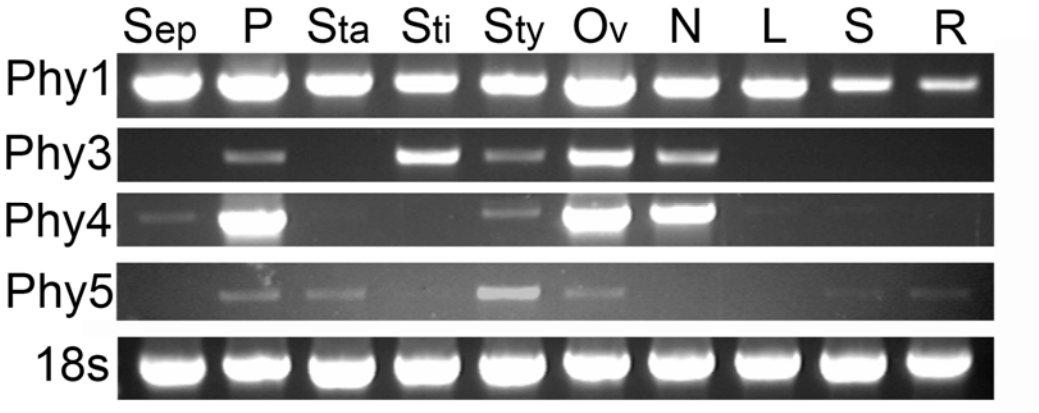

Figure 7: Expression of Petunia RNases in different flower parts. Flowers were harvested at stage 12, and dissected to obtain sepals (Sep), petals (P), stamens (Sta), stigmas (Sti), styles (Sty), ovaries (including nectaries, Ov), and nectaries (N). At the same time, leaves (L), stems (S), and roots (R) were collected. Expression of the four RNase genes was analyzed by RT-PCR. Amplification of 18S was used as control for loading. Gels are representative of at least 3 independent experiments.

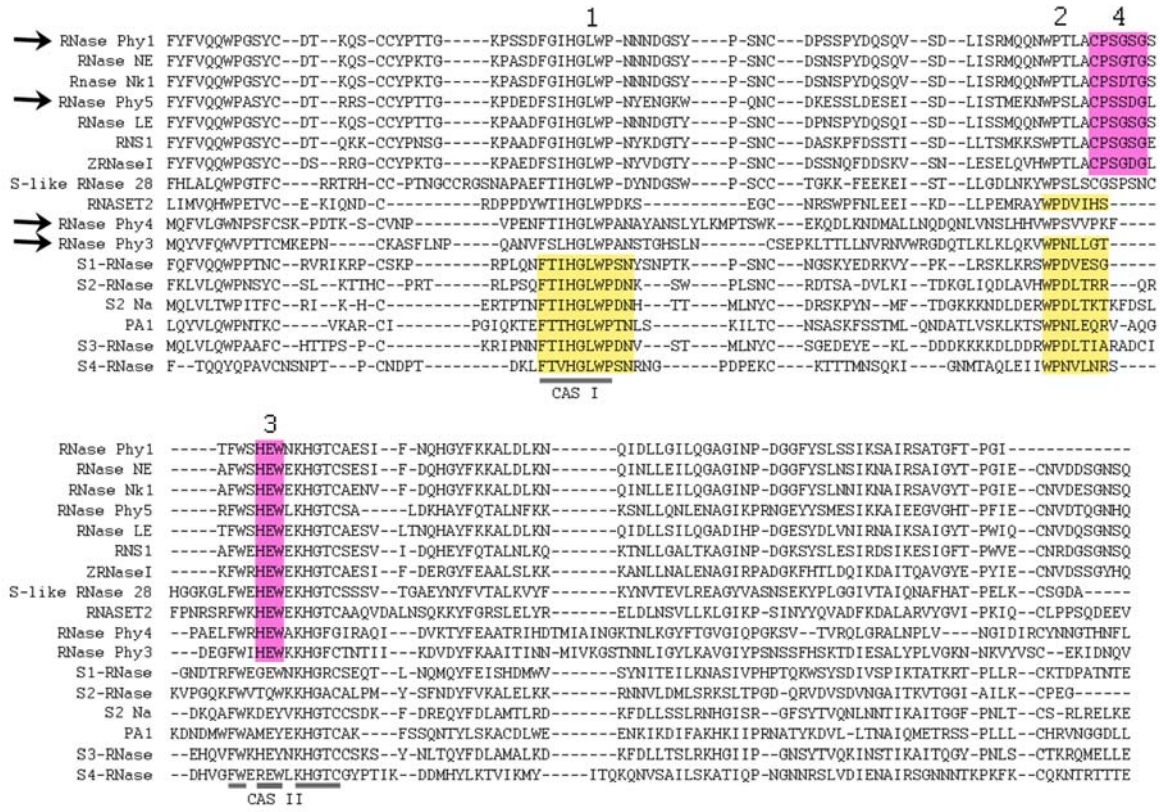

Figure 8: Presence of S- and S-like RNase-specific patterns (according to (Vieira *et al.*, 2008)) in Petunia RNases. Alignment of the Petunia RNases and representative members of the S-RNase and the S-like RNase subfamilies. Patterns 1 and 2 that define S-RNases are highlighted in yellow; S-like RNase patterns are pink. The conserved active sites (CAS) I and II, typical of RNase T2 enzymes, are indicated. Petunia RNases are indicated with arrows. Accession number of other S-like RNase proteins in the alignment are AAA21135 (RNase NE), BAA95448 (RNase Nk1), X79337 (RNase LE), P42813 (RNS1), AAC49325 (ZRNaseII), CAC50874 (S-like RNase 28); S-RNases included are BAA83479 (S1-RNase), CAA65319 (S2-RNase), AAB40027 (S2 Na), BAD11006 (PA1), AAB07492 (S3-RNase), and BAA28354 (S4-RNase). We also included NP_003721 (RNASET2) from *Homo sapiens*.

Supplemental Table 1. Primers used in this work

Cloning / RT-PCR	5' -> 3'
RNase Phy1 -Forward	GGCGTCAATATTTGCCACTTCTATG
RNase Phy1 -Reverse	CGATTCCAGGAGTAAATCCAGTTG
RNase Phy3 -Forward	CTGAATTGCTCGGAACCAAAATTAAC
RNase Phy3 -Reverse	TGACAATATTTTTGGGGGACGGC
RNase Phy4 -Forward	GTAGTAAGCCGGACACAAAGTCATGC
RNase Phy4 -Reverse	CCTGTTAGTTGTACCTGTCCCGTTC
RNase Phy5 -Forward	ATTGTGATACAAGGCGTAGTTGCTG
RNase Phy5 -Reverse	GAGAAAGGAGGGAATTCAATCTTAG
18s - Forward	ACTAATTCAGACTGTGAAACTGCGA
18s - Reverse	CTTGCTTTGAGCACTCTAATTTCT

RACE	5' -> 3'
RNase Phy3 -Forward	TGGTACGGATGAGGGGTTCTGGATACATG
RNase Phy3 -Reverse	GGACGGCCCACAACCTCTATTAGCATGAG
RNase Phy4 -Forward	GGCCAGCAAATGCATACGCGAACAGTTTG
RNase Phy4 -Reverse	CCAAGGGGTTGAGAGCTCTCCCAAGTTGAC

CHAPTER 4: Zebrafish RNase T2 genes and the evolution of secretory ribonucleases in animals

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Authors' contributions

MSH carried out the gene expression analyses, RNase activity characterization, and part of the cloning. LR and AU performed the initial cloning and characterization of alternative splicing. YW and JJE carried out the in situ hybridizations. GCM conceived of the study, and performed the phylogenetic analyses. MSH and GCM participated in the design of the study and drafted the manuscript. All authors read and approved the final manuscript.

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Abstract

Background

Members of the Ribonuclease (RNase) T2 family are common models for enzymological studies, and their evolution has been well characterized in plants. This family of acidic RNases is widespread, with members in almost all organisms including plants, animals, fungi, bacteria and even some viruses. While several biological functions have been proposed for these enzymes in plants, their role in animals is unknown. Interestingly, in vertebrates most of the biological roles of plant RNase T2 proteins are carried out by members of a different family, RNase A. Still, RNase T2 proteins are conserved in these animals.

Results

As a first step to shed light on the role of animal RNase T2 enzymes, and to understand the evolution of these proteins while co-existing with the RNase A family, we characterized RNase Dre1 and RNase Dre2, the two RNase T2 genes present in the zebrafish (*Danio rerio*) genome. These genes are expressed in most tissues examined, including high expression in all stages of embryonic development, and their expression corresponds well with the presence of acidic RNase activities in every tissue analyzed. Embryo expression seems to be a conserved characteristic of members of this family, as other plant and animal RNase T2 genes show similar high expression during embryo development. While plant RNase T2 proteins and the vertebrate RNase A family show evidences of radiation and gene sorting, vertebrate RNase T2 proteins form a monophyletic group, but there is also another monophyletic group defining a fish-specific RNase T2 clade.

Conclusions

Based on gene expression and phylogenetic analyses we propose that RNase T2 enzymes carry out a housekeeping function. This conserved biological role probably kept RNase T2 enzymes in animal genomes in spite of the presence of RNases A. A hypothetical role during embryo development is also discussed.

Background

Ribonucleases (RNases) have long been used as biochemical models of enzymology and protein folding, and also as models for molecular phylogenetic and evolutionary analyses [1] [2] [3]. The RNase A and RNase T2 families are among those better characterized. The acidic ribonuclease RNase T2 was first purified from *Aspergillus oryzae* and characterized by Sato and Egami [4]. The RNase T2 superfamily is widespread [1], with members in almost all organisms analyzed to date, including bacteria, fungi, plants, animals and even viruses. RNase T2 enzymes are secreted RNases without base specificity, and they can degrade all types of single-stranded RNA [1]. Phylogenetic analysis of this family has been carried out extensively in plants, in particular in models of evolution of gametophytic self-incompatibility [5] [6] because a subclass of the RNase T2 family, the S-RNases, is involved in this process. The T2 family has expanded and diversified in plants, and each angiosperm genome sequenced so far contains five or more genes belonging to this family (A. Meyer and G.C. MacIntosh, unpublished). These genes are classified as S-RNases or as S-like RNases, depending on whether they are involved in the self-incompatibility process or not [7]. A nutritional role as phosphate scavengers and defense roles as antibacterial, antifungal, or antiviral agents are among the functions proposed for S-like RNases [1] [7].

In animals, the vertebrate-specific RNase A superfamily has been exhaustively studied [2]. RNase A enzymes are secreted proteins with pyrimidine base-specificity that can degrade any kind of single stranded RNA, and in some cases double stranded RNA [8]. This family has also been used in a variety of evolutionary studies, from mammalian and vertebrate phylogenetics [3] [9] to analyses of evolution of novel gene functions after gene duplications [10] [11]. Among the biological functions assigned to RNase A family members are nutrition, as a phosphate and nitrogen scavenger in the gut [12], and defense, due to antibacterial and antiviral properties [13] [14]. These functions are similar to those assigned to RNase T2 members in plants. In addition, while some enzymatic differences exist between these two families, the main substrate seems to be similar.

The RNase T2 family has experienced a large expansion and diversification in plants; [5] [6](Meyer A and MacIntosh GC, unpublished), and a parallel can be drawn to the RNase A family expansion in vertebrates [9] [13]. In spite of these similarities, RNase A members have not been able to completely replace RNase T2 functions in vertebrates, since at least one gene belonging to the latter family has been found in each animal genome completely sequenced.

To gain insights on the evolution and coexistence of these RNase families we analyzed RNase T2 members found in the zebrafish (*Danio rerio*) genome. We chose this organism because its genome has been completely sequenced, and all developmental stages, from early embryo to adult, can be easily obtained. In addition, well-detailed analyses of zebrafish RNase A genes have been recently published [15] [16] [17]. Here we show that the zebrafish genome contains two RNase T2 genes. Expression of RNase T2 genes in all adult and embryo tissues suggests that this family of RNases have a housekeeping function, in contrast to the roles of RNase A, which are tissue- and stress-specific. In addition high RNase T2 embryo expression is conserved in various eukaryotes, both plant and animals, suggesting that an embryo-specific function could also be important to maintain this family's presence in vertebrates even after RNase A genes appeared.

Results

RNase T2 enzymes are present in zebrafish

Although early studies detected only faint RNase activity in fish organ extracts [18], an RNase T2 with acidic pH preference was recently isolated from salmon liver [19]. To identify RNase activities in zebrafish extracts we used a standard *in gel* activity assay that allows size separation of different proteins with RNase activity, as well as characterization of pH preference. Adult zebrafish of mixed sexes were separated into "body" (mostly muscle, skin and skeleton), "head" (which included skull, muscle, skin, brain, eyes among other tissues) and "guts" (which included most internal organs such as intestine, liver, heart, sexual organs). Crude extracts were then analyzed for RNase activities (Figure 1A). At

neutral pH we identified only weak activities in the molecular weight range of RNase A (12-18 kDa). However, at an acidic pH we also observed stronger activities in the 20-30 kDa range that could correspond to RNase T2 enzymes. While body and head extracts clearly showed all activities, gut extracts did not show any detectable RNase activity in the conditions assayed. This result is not due to general protein degradation since protein integrity seems evident in a Coomassie stained SDS-PAGE (Figure 1B).

Identification of several bands in the RNase A range was consistent with the four RNase A genes found in the zebrafish genome [15] [16]. Thus, several bands in the RNase T2 range suggested that the zebrafish genome also contained more than one RNase T2 gene. A BLASTP [20] search against the protein prediction database of the current zebrafish genome assembly (Zv7) using the RNase Ok2 sequence from salmon [19] identified two proteins with homology to RNase T2 enzymes, one located in chromosome 15 and the other in chromosome 13. Additional searches using these two proteins (using TBLASTN), or the corresponding nucleotide sequences (using BLASTN) against the full genome assembly and available ESTs failed to identify any additional sequences corresponding to RNase T2 homologs.

The two proteins contain conserved amino acid sequences (CAS I and CAS II, Figure 2A) characteristic of the RNase T2 family, which include the His residues (* in Figure 2) essential for RNase activity. They also have conserved Cys residues important for establishment of tertiary structure, and other residues conserved in most RNase T2 homologs [1]. We named these enzymes RNase Dre1 (chromosome 15) and RNase Dre2 (chromosome 13). Molecular weights of the predicted mature peptides are 23.7 kDa and 27.3 kDa for the two forms of RNase Dre1 (see below) and 25.4 kDa for RNase Dre2. In addition, four N-glycosylation sites are predicted for RNase Dre1 and two for RNase Dre2. Incomplete glycosylation of the proteins at these sites could account for all the bands in the 20-30 kDa range observed in the activity gels shown in Figure 1.

Using RT-PCR we cloned cDNAs corresponding to both genes, and confirmed the sequence of the predicted proteins. Both predicted proteins appear to have signal peptides that may direct them to the secretory pathway. Based on

the sequence of the predicted mature peptides, the identity between RNase Dre1 and RNase Dre2 is only 31%; in fact RNase Dre2 is more similar to RNASET2, the human RNase T2 homolog (44% identity), while RNase Dre1 is only 26% identical to RNASET2. On the other hand, both proteins are 22% (RNase Dre2) and 19% (RNase Dre1) identical to RNase T2 from *Aspergillus oryzae*, the prototypic RNase of the family [1]. Analysis of genomic organization (Figure 2B) showed that *RNase Dre1* has 9 exons, while *RNase Dre2* has 14, although the coding regions for both genes are contained in 9 exons.

During cloning we observed that *RNase Dre1* RT-PCR showed two different bands, with less than 100 bp difference in size. Cloning and sequencing of individual bands (See Additional File 1) resulted in the identification of an alternative splicing variant. The presence of the alternative exon of 74 nt in the mRNA (black box in Figure 2B) results in a longer mRNA. The expression of both mRNA species was confirmed by Northern blots (not shown). This extra exon also results in a change in the open reading frame, which in turn changes the start codon position. Thus, the short mRNA species produces a longer peptide, while the long mRNA produces a shorter one. This change only affects the signal peptide (Figure 2A). Subcellular localization prediction programs predict that both isoforms of RNase Dre1, as well as RNase Dre2, contain a signal peptide that target the proteins to the secretory pathway, as is the norm for most members of the RNase T2 family. However, the putative signal peptide in the shorter RNase Dre1 protein includes sequences that are highly conserved in all RNase T2 proteins, suggesting that this putative peptide might not be cleaved, or that it could result in a non-functional protein.

RNase Dre1 and *RNase Dre2* are expressed in adult and embryo tissues

To determine when and where the two RNase T2 genes were expressed, we used semi-quantitative RT-PCR. Adult fish tissues were dissected and total RNA was isolated. To be able to compare *RNase Dre1* and *RNase Dre2* expression with that of RNase A genes, we used the same tissues utilized in the analysis by Cho and Zhang [16]. These included brain, eye, heart, liver, gut, muscle, ovary, testis and skin. *RNase Dre2* was expressed in all organs, and a

stronger signal was detected in reproductive organs (Figure 3A). *RNase Dre1* was not detected in liver and the signal was weak in eye, ovary and skin under the conditions of our experiment. These expression patterns contrast with those observed for RNase A homologs, which are expressed almost exclusively in liver and gut tissues, and weakly in heart (*Dr-RNase 1*, *Dr-RNase 2* and *Dr-RNase 3*; [16]), or brain (*Zf-RNase-1*; [17]). It is important to note that while expression of *RNase Dre1* and *RNase Dre2* is clearly detected in several internal organs, RNase activity is not easily detected in "guts" extracts (Figure 1A). Further characterization of these extracts indicated that although RNase activities are present, they are degraded by proteases that seem to have a certain amount of specificity, since most proteins in gut extracts seem unaltered after Coomassie blue staining (Figure 1 and data not shown).

Analysis of expression in whole embryos during developmental stages was also performed (Figure 3B). Strong expression of *RNase Dre1* was observed during all developmental stages analyzed (2, 5, 12, 24, 72 h after fertilization), with a peak of expression at 72 hours. *RNase Dre2* was also detected in all developmental stages and peaked at 72 h, although the signal was lower at 5 and 12 h. In contrast, only one of the RNase A homologs, *Zf-RNase-1*, is expressed in early embryo tissues [16] [17]. Analysis of RNase activity in embryo extracts showed a pattern consistent with mRNA expression results. Only RNase activities in the 20-30 kDa range were detected, and only in acidic conditions (Figure 3C).

To further confirm that *RNase Dre1* and *RNase Dre2* are expressed in zebrafish embryos, we performed *in situ* hybridization analyses (Figure 4). As expected, both mRNAs were detected in all the embryo stages studied. At the one cell stage (Figure 4, panels A-B) transcripts corresponding to both RNases localized mainly to the animal pole, or the part of the cell that will contribute to the embryo proper. It was also possible to observe RNA projections in structures that resemble cytoskeletal arrangements extending from the animal pole toward the vegetal pole associated with axial streaming of ooplasm [21]. These structures could correspond to RNA being recruited to the embryo from the extraembryonic yolky cytoplasm [22]. At the 16-cell stage (Figure 4, panels C-D) both RNAs gave

a strong signal in blastomeres. Twenty six-hour embryos (Figure 4, panels E-F) showed strong expression throughout the embryo, and only *RNase Dre2* showed weak expression in yolk, with both RNases most highly expressed in eyes.

RNase T2 enzymes are also expressed in embryos of other organisms

To investigate whether other RNase T2 homologs also have a role during embryo development we analyzed expression of genes belonging to this family using available microarray data from public databases. *CeRNS*, the only RNase T2 homolog gene in the nematode *Caenorhabditis elegans* genome is also expressed during embryo development (Figure 5A). Microarray data also indicated that *CeRNS* is not expressed in adult tissues (not shown). In situ hybridization results obtained from The Nematode Expression Pattern DataBase (Tadasu Shin-i and Yuji Kohara, unpublished, http://nematode.lab.nig.ac.jp/) confirmed the expression pattern of *CeRNS* obtained from microarray databases. *CeRNS* embryo expression seems to be ubiquitous, as is the case for *RNase Dre1* and *RNase Dre2.* In addition, analysis of expression data representing 61 mouse tissues [23] showed that the only RNase T2 homolog present in the mouse genome was also detected in embryonic samples (not shown). Remarkably, embryo expression is not limited to animals. According to microarray data [24], *RNS1*, one of five members of the RNase T2 family present in the plant *Arabidopsis thaliana*, is one of the most highly expressed genes (98-99 percentile) during embryo development (Figure 5B).

RNase Dre1 represents a gene duplication present only in ray-finned and cartilaginous fishes

In order to understand the evolution of RNase T2 genes in fish and other animals, we searched for sequences belonging to this family in EST and protein databases. We also analyzed the fully sequenced genomes of the ray-finned fishes medaka (*Oryzias latipes*), spotted green pufferfish (*Tetraodon nigroviridis*), and fugu (*Takifugu rubripes*). In these three genomes we identified two genes in each species belonging to the RNase T2 family, as in the zebrafish genome, although only one in each case was also represented in EST collections.

Additional sequences belonging to this family were found in EST collections for other fish species, including sharks, lamprey and hagfish (see Figure 6 and Additional File 2 for full list of species).

Among the many RNase T2 sequences available from other organisms, we selected several sequences from vertebrates (platypus, opossum, mouse, human, chicken, frog), an Urochordate, a Cephalochordate, an Echinoderm, nematodes, a trematode and an insect to generate a protein Neighbor-Joining tree of animal RNases (Figure 6). Proteins from plants (RNase LE and RNS1), bacteria (RNase I and RNase AhyI), protozoa (RNase DdI) and fungi (RNase T2 and RNase Rh) were included to identify the relationship of animal RNases with other proteins in the RNase T2 superfamily.

The tree allowed us to make several inferences on the evolution of the RNase T2 family in animals. Fish RNases cluster in two well defined clades, one represented by RNase Dre1 and the other by RNase Dre2 (red and yellow boxes in Figure 6). Evidence for genes belonging to the two clades was found in all fully sequenced fish genomes by BLAST searches, although only those with EST support were included in the tree shown in Figure 6, because a clear gene model for the other genes was not available. All the fish species for which full genome sequence is available contain only one gene from each clade. Similarly, only one sequence for each clade was found in several fish EST collections with the exception of the brook trout (*Salvelinus fontinalis*), in which a recent duplication gave rise to two copies of the RNase Dre1 homolog (RNase Sfo1 and RNase Sfo3 in Figure 6).

The presence of genes from the two clades in Chondrichthyes (cartilaginous fishes) and Actinopterygii (ray-finned fishes) indicates an ancient origin for the gene duplication that gave rise to these two clades, with the two genes present at least in the last common ancestor of the two classes more than 400 MYA [25]. Analysis of sequence data from earlier Chordata, including sea squirt (*Ciona intestinalis*), amphioxus (*Branchiostoma floridae*), hagfish (*Eptatretus burgeri*), and lamprey (*Petromyzon marinus*) identified only one gene belonging to the RNase T2 family in each species. Since the amphioxus genome has been completely sequenced [26], the presence of only one gene would

indicate that the gene duplication occurred after the separation of the Cephalochordata from the main Chordata stem. Since genome coverage for the other species is limited, the exact timing for the gene duplication leading to the two RNase clades cannot be precisely determined. Moderate bootstrap support (76%) for the RNase Dre2 clade suggests that the duplication predated the divergence of lampreys and hagfishes from jawed vertebrates. However, this result could also mean that the genes in the RNase Dre2 clade conserved more ancestral characteristics than RNase Dre1 after duplication.

Genes belonging to the RNase Dre2 clade were found in all vertebrates analyzed, from hagfish to human. However, RNase Dre1 clade genes were found only in cartilaginous and bony fishes, but not in other vertebrates. Exhaustive analysis of the fully sequenced human and mouse genomes failed to identify RNase Dre1 genes. Interestingly, an RNase T2 pseudogene was found in each of these two genomes (not shown), but in both cases the pseudogene also belonged to the RNase Dre2 clade (the human pseudogene presented 84% identity with the human RNASE T2 protein, while the mouse pseudogene had 63% identity with mouse RNase T2 protein RNase Mmu2). These results suggest that the RNase Dre1 gene was present at least in the last common ancestor of Actinopterygii (ray-finned fishes) and Sarcopterygii (lobe-finned fishes and tetrapods) but was lost in Tetrapods after they diverged.

The recent characterization of RNase Ok2 from salmon [19] showed that in this protein, a commonly conserved His residue in CAS II (His104 in RNase Rh, Tyr102 in RNase Dre2) is mutated to Tyr. This change most likely affects the catalytic properties of the enzyme and results in lower specific activity [19]. Our results showed that the same mutation is found in all Teleostei (modern ray-finned fishes) (Figure 7), but not in sturgeon (RNase Atr2), sea lamprey (RNase Pma2), and dogfish shark (RNase Sac2), nor in other vertebrates outside fish, suggesting that the mutation appeared and was fixed at the base of this taxon or after the separation of Actinopterygii (ray-finned fishes) and Sarcopterygii (Coelacanths and tetrapods). Interestingly, the same mutation was found in RNase Ebu2 from inshore hagfish, and it is most likely the result of an independent mutation that was fixed in this species (or near taxa).

Remarkably, all analyzed genes belonging to the RNase Dre1 clade also showed mutations in the same position (Figure 7). In this case the canonical His residue characteristic of other RNase T2 enzymes is replaced by a series of charged or polar amino acids: Glu, Asp, Gln, or Asn. Since all genes in this subfamily have substitutions in this position, the loss of the His residue seems to have happened soon after the duplication event that gave rise to the RNase Dre1 clade. Although no mutagenesis experiments have been carried out to show the effect of these mutations, some S-RNases have similar substitutions (Figure 7). S-RNases have low specific activity compared with other RNase T2 enzymes, and such characteristic has been attributed to the lack of this particular His residue [27]; however, they are still active, and this activity is essential for their biological function. Since the amino acid substitutions in this position in S-RNases and RNase Dre1 homologs are the same, it is expected that the changes observed in RNase Dre1 homologs also reduce, but do not eliminate, the specific activity of these enzymes.

Discussion

Ribonucleases from the RNase A and RNase T2 family have been frequently used as models for the study of evolution of gene function. These two types of RNases have similar enzymatic activity and substrate preferences, both being endoribonucleases that mainly hydrolyze bulk single stranded RNA. Both families are also found mainly in extracellular space or associated with the secretory pathway. While the RNase A family is vertebrate specific [16], the RNase T2 family is widespread and members of this family have been found in almost all eukaryotic and many prokaryotic genomes [1] [28]. Thus, in spite of this seemingly redundant activity, both enzyme families coexist in vertebrates. Evolution and biological function of RNase T2 proteins have been studied mostly in plants [5] [6] [7], although recent reports of an association of human RNASE T2 with cancer have spiked interest in this protein [29] [30].

In this work we characterized the two RNase T2 genes present in the zebrafish genome. A recent analysis of RNase A genes from this fish suggests

that the available genome sequence may not be complete, as at least one RNase A gene found in cDNA libraries is not found in the genome [15] [16] [31]. However, based on the lack of any other RNase T2 sequence in zebrafish cDNA collections, the presence of only two genes in the other fully sequenced fish genomes, and our phylogenetic analysis, we feel confident that only two RNase T2 genes are present in the zebrafish genome.

We were able to detect ribonuclease activities in zebrafish extracts that show the molecular weight and enzymatic properties expected for the proteins encoded by *RNase Dre1* and *RNase Dre2*. In addition, the conservation of the active site residues, and the high sequence similarity between the zebrafish RNases and RNase Ok2 (more than 50% identity between RNase Dre2 and RNase Ok2), which was shown to be an active ribonuclease by purification from salmon liver [19], strongly suggest that RNase Dre1 and RNase Dre2 are active ribonucleases.

Fish seem unusual in that all species analyzed have two genes belonging to the RNase T2 family, whereas all other animals have only one. Genomic data indicate that a whole genome duplication event (WGD) occurred in the fish lineage after the separation of teleosts from the main tetrapod stem. This WGD explains the occurrence of many ray-finned fish-specific gene duplications [32] [33]. However, this WGD is proposed to have occurred after the separation of the Acipenseriformes and the Semionotiformes from the lineage leading to teleost fish, but before the divergence of Osteoglossiformes [32]. Thus, the gene duplication event that gave rise to both RNase T2 genes present in fish genomes cannot correspond to this ray-finned fish-specific WGD, since genes corresponding to the RNase Dre1 and RNase Dre2 clades were found in sturgeons (ray-finned fishes but not Teleostei) and sharks (Chondrichthyes).

In contrast, the lack of RNase Dre1 orthologs in all tetrapods indicates that this gene was lost in this lineage soon after the separation from the Actinopterigii. Moreover, any duplicated gene produced by the WGD that occurred in the fish lineage was also lost. Interestingly, according to Cho and Zhang [16], RNase A genes may have appeared in the chordate lineage in the last common ancestor of these two groups. While the success of this new gene family was mixed in ray-

finned fish (zebrafish has 4 RNase A genes, but fugu and Tetraodon seem to have none), it has been successfully maintained and underwent a large diversification in tetrapods. In plants, where no RNase A genes exist, RNase T2 genes have radiated and diversified to a greater extent, in a way similar to that observed for the RNase A family in animals (A. Meyer and G. MacIntosh, unpublished). Thus, it is tempting to hypothesize that the presence of RNase A genes influenced the evolution of the RNase T2 family in ray-finned fish and tetrapods (Figure 8).

This hypothesis was supported by a series of observations. Plant RNase T2 genes and vertebrate RNase A genes show patterns of gene sorting [9] [16](A. Meyer and G. MacIntosh, unpublished) such as the presence of different gene numbers in different species and the lack of clear orthologs among species. In contrast, vertebrate RNase T2 genes form two monophyletic groups, one exclusive to fish, and the other including all vertebrates (Figure 6).

The functions acquired by duplicated genes after extensive radiation seem to be similar for the two types of enzymes in plants and animals; i.e., the biological roles assigned to many RNase A proteins in animals are similar to those of RNase T2 proteins in plants. For example, several members of the RNase A family have antimicrobial properties. Eosinophil associated RNases have antiviral (RNase 2 and RNase 3 in humans) and antibacterial (RNase 3) function, and angiogenin and RNase 7 have antibacterial and antifungal activities [14]. Similarly, plant RNase T2 proteins inhibit hyphal elongation of the pathogenic oomycete *Phytophthora parasitica* [34], and are induced by viral and fungal pathogens [35] [36]. In addition, both animal RNase A and plant RNase T2 enzymes have cytotoxic properties, for example frog oocyte RNases used as anticancer drugs [37], and flower S-RNases that reject pollen during self-incompatible pollination [38]. The cytotoxic properties of these enzymes are probably as a consequence of their role in defense, as in the case of frog oocyte RNases [39], or have evolved from a defensive role, as in the case of S-RNases [7]. A nutritional role has also been proposed for plant RNase T2 and animal RNase A enzymes. RNase A is secreted into the mammalian intestine where it helps digest RNA from gut bacteria to recover nutrients [12]; while expression of

plant RNase T2 enzymes is induced when phosphate in the soil is limited [40] [41] [42] as part of a phosphate scavenging system [43].

Finally, most plant RNase T2 enzymes and vertebrate RNase A enzymes show strong tissue specificity and lack of expression in early embryos, suggesting that they are involved in immune and stress responses rather than having a housekeeping role [16]. On the other hand, animal RNase T2 enzymes, and a few plant ones like Arabidopsis RNS2 [40] (A. Meyer and G. MacIntosh, unpublished), seem to be constitutively expressed, suggesting that they could have a housekeeping function. This function, conserved through evolution, could be responsible for the conservation of the RNase T2 family in animals, in spite of the presence of RNase A.

We hypothesize that the role of RNase T2 enzymes could be to recycle bulk RNA (mostly rRNA) throughout the life of the cell, and not only in times of nutrient deprivation as has been proposed before. RNA is an important source of P and N, and turnover of this molecule should be important for P and N homeostasis. Accordingly, some RNase T2 enzymes have been found in intracellular compartments, supporting the idea of a role recycling RNA in normal cells. For example, human RNASET2 has been found to accumulate in the lysosome [44], while Arabidopsis RNS2 is present in intracellular fractions, probably associated to the vacuole or the ER [45]. In the case of zebrafish RNase Dre1 and RNase Dre2 the localization is unknown, but subcellular localization predictions using different programs (see Material and Methods) indicate either extracellular or microsomal/lysosomal localization, almost identical to predictions for the human enzyme. It is interesting to note that the alternative splicing observed for RNase Dre1 alters the protein's signal peptide, opening the possibility that this protein localizes to different subcellular compartments. Alternative processing resulting in different subcellular localizations has already been described for tomato ribonucleases [46] [47].

The high level of expression of RNase T2 enzymes in embryonic tissues is also notable. This pattern could also be a consequence of the proposed housekeeping role for RNase T2s. The high metabolic activity of embryos could demand a high level of RNase activity to process cellular material as it is being

renewed. Alternatively, we could look for other explanations for the embryonic role of RNase T2 enzymes. A highly speculative but attractive idea is that secreted RNases can control the activity of small RNAs [48].

Following this rationale, we could speculate that the embryonic role of RNase T2 enzymes is to shield embryonic tissues from unwanted small RNAs. In plants, RNA silencing is reset in each generation [49]. This property of silencing was shown for virus induced gene silencing (VIGS) and posttranscriptional gene silencing (PTGS). Arabidopsis *RNS1* is one of the most highly expressed genes during all the stages of embryo development. Importantly, RNS1 is secreted to the apoplastic space [45]. It has been shown that the outer integument of the developing seed can provide a symplastic route for transport from maternal tissues to the developing seed, but the transfer between the outer integument and the inner integument and between the integument and the embryo are apoplastic [50] [51]. Thus, any RNA signal would have to travel through the apoplast to reach the embryo would find a barrier due to accumulation of RNS1. In the nematode *Caenorhabditis elegans* it has been shown that phenotypes induced by RNAi can last only for two or three generations [52], and only a small subset of genes (13/171) could be inheritably silenced for longer periods of time [53]. These results suggest that transmission of silencing from maternal to embryonic tissues could be regulated also in animals. In this context, secreted RNases would form a RNA surveillance field [54], that stops the spreading of small RNAs.

In summary, it seems possible that the emergence of RNase A affected the evolution of RNase T2 proteins in animals. The smaller size of RNase A proteins, which could be more energetically favorable, could favor the use of this protein instead of RNase T2 proteins for defense roles in animals. However, RNase T2 proteins have not been completely replaced in animals, most likely because they also have a housekeeping function in an intracellular compartment that cannot be carried out by RNase A.

Conclusions

The zebrafish genome contains two RNase T2 genes, *RNase Dre1* and *RNase Dre2*. These genes are part of two phylogenetic clades, one conserved in all chordates (the RNase Dre2 clade), and another fish-specific (the RNase Dre1 clade). Expression analyses indicate that *RNase Dre2* is present in all tissues and developmental stages in zebrafish, suggesting a housekeeping role for these enzymes. This idea is further supported by the conservation of RNase T2 genes in all the genomes analyzed. Analyses of the evolution of the RNase T2 family in animals, and comparisons with the evolution of RNase T2 in plants and RNase A in vertebrates suggest that the emergence of RNase A affected the evolution of RNase T2 proteins in animals.

Materials and Methods

Database searches and sequence identifications

Identification of RNase T2 genes was done by BLAST searches in the zebrafish (*Danio rerio*) genome (version Zv7, available through the National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/). Analyses of the medaka (*Oryzias latipes*), spotted green pufferfish (*Tetraodon nigroviridis*), and other full genomes was also performed using NCBI resources. Analysis of the fugu (*Takifugu rubripes*) genome was performed using Assembly Release 4 from the Fugu BLAST server (http://fugu.biology.qmul.ac.uk/blast/). Expressed sequence tag (EST) sequences and protein sequences were also obtained by BLAST searches of the NCBI EST-other and non-redundant databases respectively. Analysis of genome organization for the RNase Dre1 and RNase Dre2 genes was done using contigs obtained by combining information from cDNAs cloned by our laboratory (supplementary dataset) and ESTs obtained from NCBI EST-other.

Prediction of signal peptides and subcellular localization was carried out using PSORT [55], WoLF PSORT [56] and SignalP and TargetP [57].

Arabidopsis microarray data were obtained from the Arabidopsis information Resource (TAIR) database. Mouse microarray data were obtained from the Genomics Institute of the Novartis Research Foundation SymAtlas

(http://symatlas.gnf.org/SymAtlas/). Nematode microarray data were obtained from WormBase (http://www.wormbase.org/). In all cases, normalized data were used, and values belonging to the same experiment set were compared.

Zebrafish samples preparation and cDNA cloning and RT-PCR

Wild zebrafish and laboratory strain WIC were used in our experiments. *RNase Dre1* and *RNase Dre2* cDNAs were amplified from 48hr post-fertilization embryo RNA using primers designed based on database sequences. Embryos were broken by forcing the sacs through a syringe fitted with a sterile needle prior to extraction. Total RNA was purified using the TRIzol reagent according to the manufactures directions (Invitrogen), and cDNAs were synthesized using the iSCRIPT kit (BioRAD). PCR was performed using the following primers: *RNase Dre1*F 5'CGCGATATCACAGGCTGTTTGTTACTGAC3', *RNase Dre1*R 5'CGCCCATGGGCGCTTGCACCGGTGGGTAATA3', *RNase Dre2*F 5'CGCGATATCACAGACTCTCAGAACAGACG3' and *RNase Dre2*R 5'CGCCCATGGGGTTACATGGCTCATGAGGA3'. During cloning, we amplified two PCR products corresponding to *RNase Dre1*. Both bands from *RNase Dre1* and a single band from *RNase Dre2* amplification were gel purified using a Gel Purification kit (Promega). The genes were cloned using the pGEM-T Easy kit (Promega) and sequenced. Sequencing reactions were performed at the DNA Facility at Iowa State University using T7 and SP6 primers.

Expression analyses were performed using semiquantitative RT-PCR. Adult fish were dissected into the following organs: brain, eyes, heart, liver, gut (digestive system), muscle, ovary, testis, and skin. Excluding reproductive organs, all tissue samples came from fish of both sexes. RNA was extracted and cDNA was generated as described previously. PCR amplification was done using GoTAQ Green Master Mix (Promega). The cDNA corresponding to the ribosomal protein p70 was used as loading control for RT-PCR. The primer sequences used for p70 were p70/6sk-r1 5'AGCTTGCCGCCCGTCTGAAA3', and p70/6sk-f1 5'CATGGCGACGGTGCGTTCAT3'. Primer sequences for *RNase Dre1* and *RNase Dre2* are the same as those listed above. Gels were stained with ethidium bromide and visualized using the NIH Image program. All

experiments were performed a minimum of 3 times and a representative sample was chosen for each figure.

RNase Activity

Adult fish of both sexes were dissected into the following sections: head, including all tissues above the heart; body, including skin, muscle, and bones of the main body; and gut, including the digestive and reproductive systems. Proteins were extracted from each section following the method used by MacIntosh *et al.* [58]. The protein extraction protocol was modified by eliminating β-mercaptoethanol and polyvinylpolypyrrolidone from the extraction buffer. Protein was quantified according to the Bradford method. RNase activity was determined by an *in gel* assay according to Yen and Green [59], using high molecular weight RNA purified from commercial torula (yeast) RNA (SIGMA). One hundred µg of protein were run for each sample. Gels were incubated in 0.1M Tris-HCl at either pH 6.0 or pH 7.0 as identified in the figure. In parallel, SDS-PAGE gels were run using 100 µg of protein to verify loading amounts and protein quality. All experiments were performed a minimum of 3 times and a representative sample was chosen for each figure.

In situ hybridizations

Whole-mount in situ hybridizations were performed as described by Essner *et al.* [60], using 1-cell (~30 min. post fertilization), 16-cell (~ 1.5 h post fertilization) and prim 6 (~26 h post fertilization) embryos [61]. RNA probes were prepared *in vitro* transcription from linearized templates of *RNaseDre1* and *RNaseDre2* cDNA in the pGEM T-Easy vector.

Phylogenetic analysis

Protein sequences were aligned using ClustalW2 [62] followed by manual adjustments. PAUP 4.0 software [63] was used for phylogenetic analyses. Phylogenetic trees were constructed using the Neighbor-Joining tree method [64] with 1,000 bootstrap replications.

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Figures

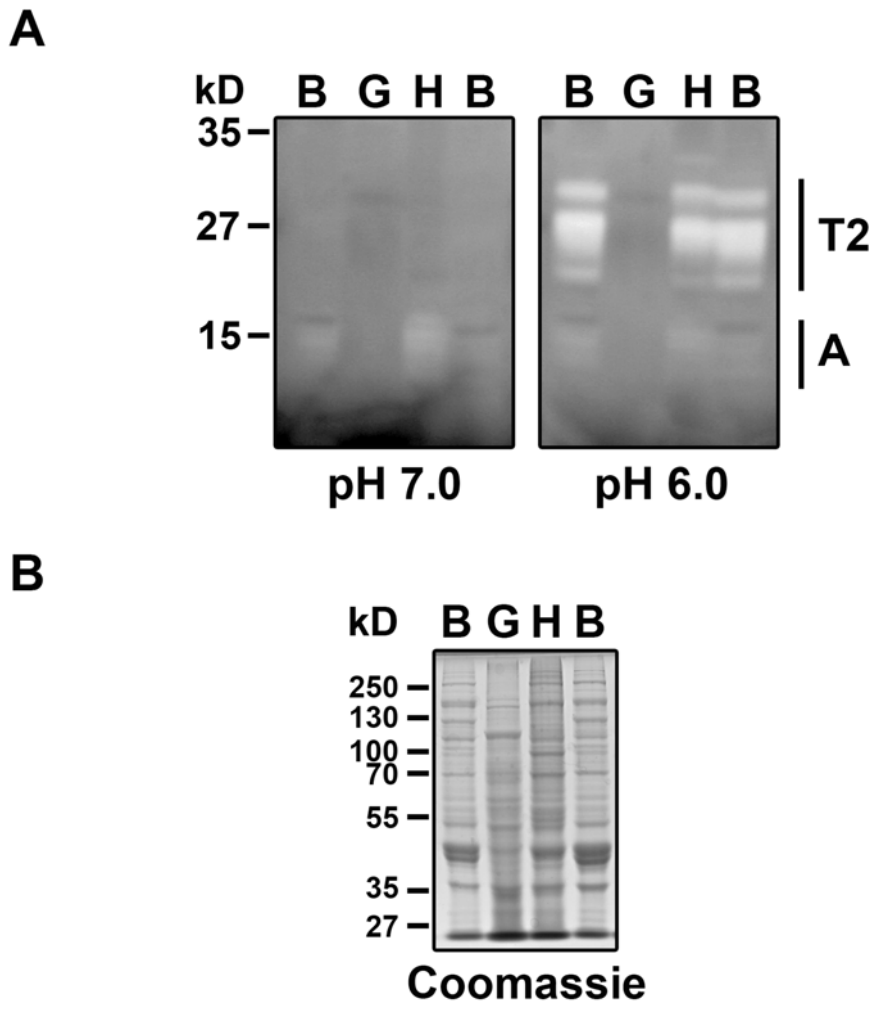

Figure 1 - Characterization of zebrafish RNases

A) Ribonuclease activities present in zebrafish extracts. Adult zebrafish extracts were analyzed in an *in gel* RNase assay at two different pHs. Adult zebrafish of mixed sexes were separated into “body” (B, mostly muscle, skin and skeleton), “head” (H, which included skull, muscle, skin, brain, eyes among other tissues) and “gut” (G, which included most internal organs such as gut, liver, sexual organs, heart). The size range for RNase T2 and RNase A proteins is indicated. **B**) Same samples as in A, analyzed by SDS-PAGE and stained with Coomassie Blue. One hundred µg of protein per lane were analyzed in both types of gels.

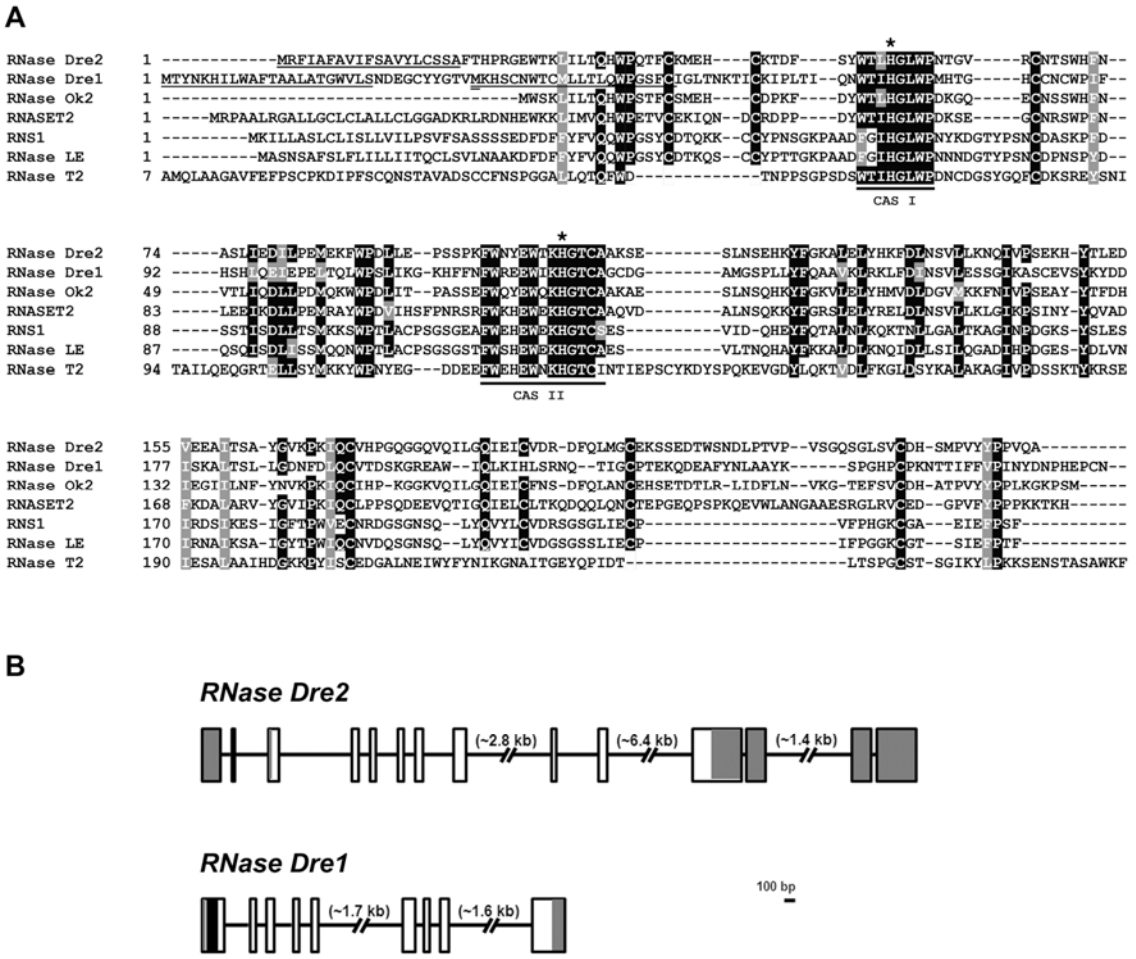

Figure 2 - The zebrafish genome contains two RNase T2 genes

A) Alignment of the two predicted RNase T2 proteins (RNase Dre1 and RNase Dre2) present in the zebrafish genome with RNase T2 proteins from salmon (RNase Ok2), human (RNASET2), *Arabidopsis thaliana* (RNS1), tomato (RNase LE) and *Aspergillus oryzae* (RNase T2). Residues conserved in all RNase T2 enzymes are highlighted. CAS I and CAS II, conserved active-site segments that contain the two Histidines (*) involved in catalysis. The predicted signal peptides for RNase Dre1 and RNase DRe2 are underlined, and the alternative starting Methionine in RNase Dre1 is double-underlined. **B**) Structure of the two RNase T2 genes identified in the zebrafish genome. The intron-exon structure was obtained by comparison of the sequences obtained from direct cloning and sequencing of cDNA with the publish sequence of genomic DNA. Boxes indicate exons, lines indicate introns. Gray shading indicates untranslated regions, white indicates coding region, and black marks the region that undergoes alternative splicing in *RNase Dre1*. Gene accession numbers for the zebrafish proteins are FJ460212 for RNase Dre2 and FJ460210 and FJ460211 for the two different splicing variants of RNase Dre1.

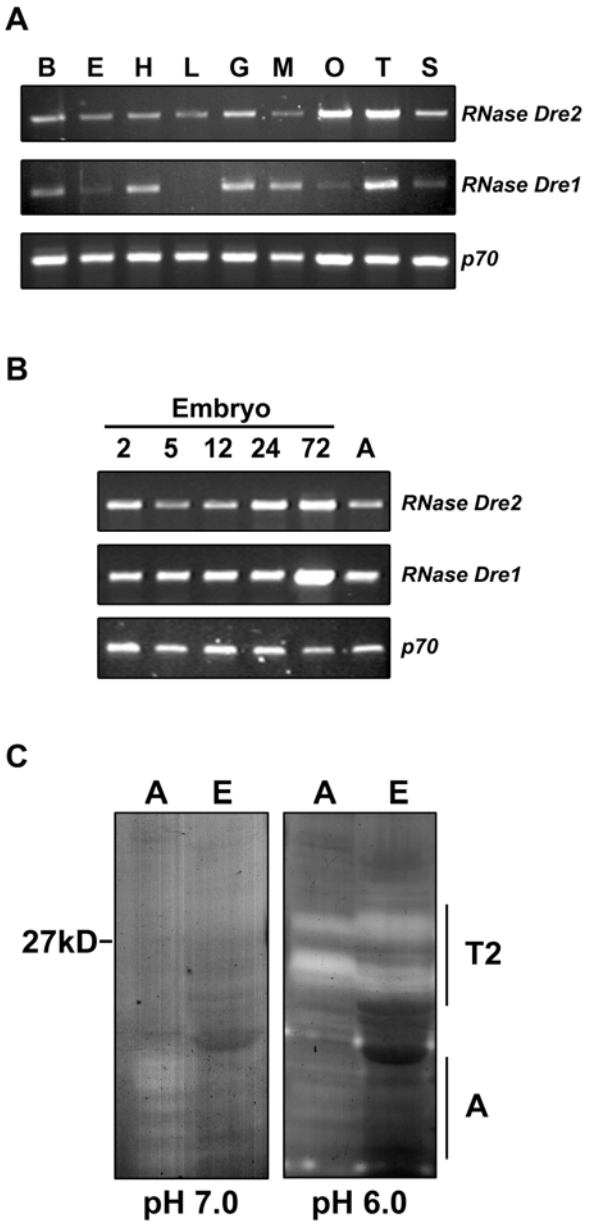

Figure 3 - Expression of zebrafish *RNase Dre1* and *RNase Dre2*

A) RT-PCR analysis of expression of *RNase Dre2* and *RNase Dre1* in adult tissues: B, brain; E, eye; H, heart; L, liver; G, gut; M, muscle; O, ovary; T, testis; S, skin. p70 was used as control for loading. **B**) RT-PCR analysis of expression of *RNase Dre2* and *RNase Dre1* in embryos at different times (in days) after fertilization. **C**) Ribonuclease activities present in zebrafish embryos (E) and adults (A) analyzed by *in gel* activity assay as in Figure 1.

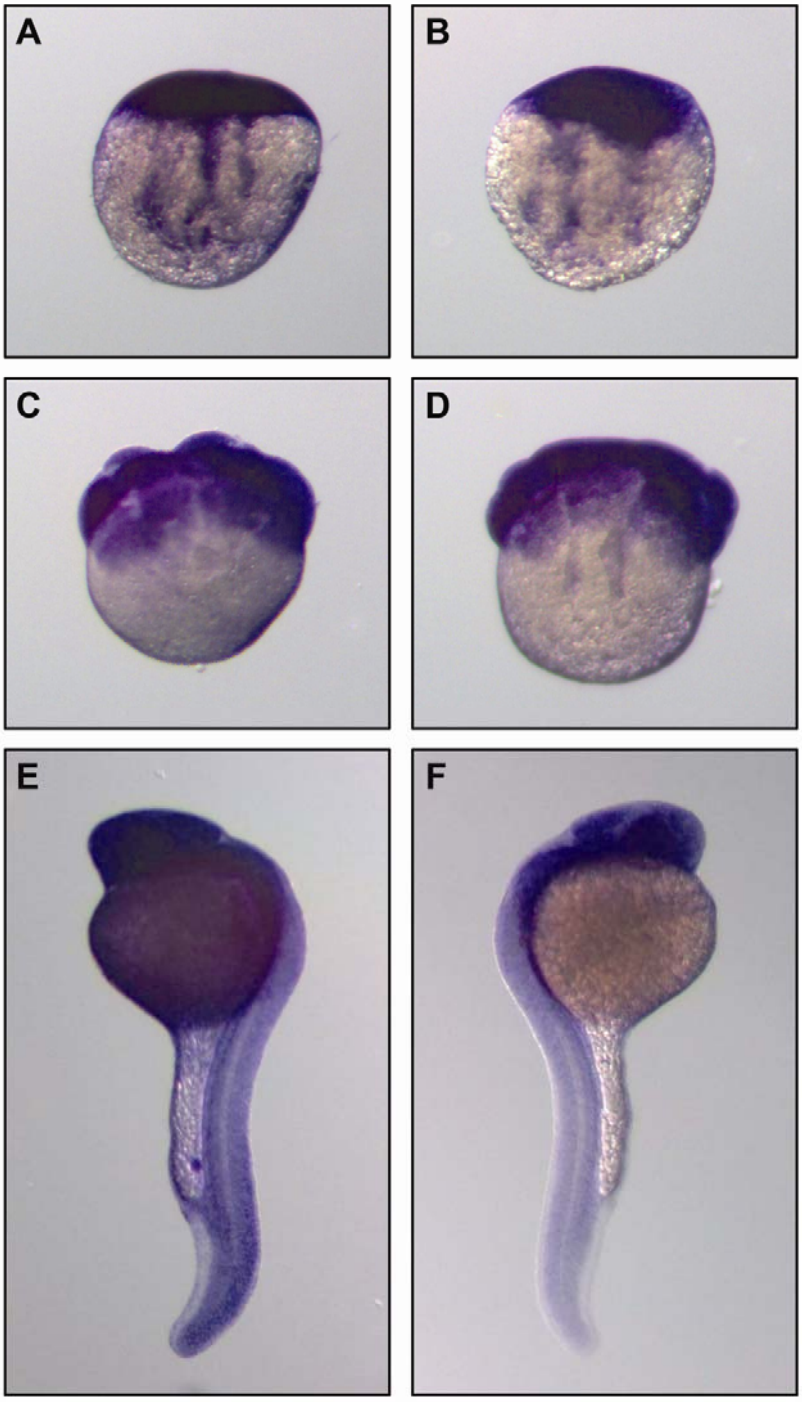

Figure 4 - Localization of *RNase Dre1* and *RNase Dre2* expression in zebrafish embryos

Whole-mount *in situ* hybridization analysis was performed in embryos at the 1-cell stage (A, B), 16-cell stage (C, D) and prim 6 stage (E, F). Left panels, *RNase Dre2* probe; right panels, *RNase Dre1* probe.

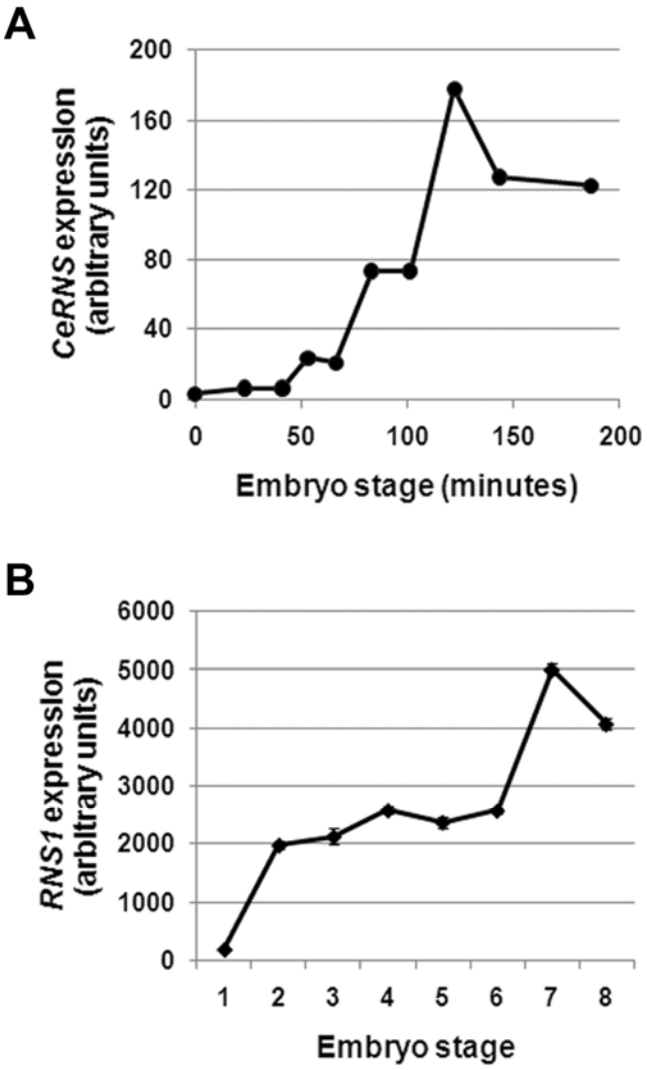

Figure 5 - RNase T2 genes are expressed in embryos in other organisms

Expression of RNase T2 genes during embryo development in the nematode *Caenorhabditis elegans* (**A**) and the plant *Arabidopsis thaliana* (**B**). Expression data were obtained from public microarray databases. Values indicate arbitrary fluorescence intensity units after normalization. **A**) Stages of nematode embryo development indicated as minutes after fertilization. **B**) Arabidopsis embryo stages: 1, globular; 2, heart; 3, triangle; 4, torpedo; 5, curly cotyledon; 6, curly cotyledon 2; 7, mature cotyledon; 8, green cotyledon.

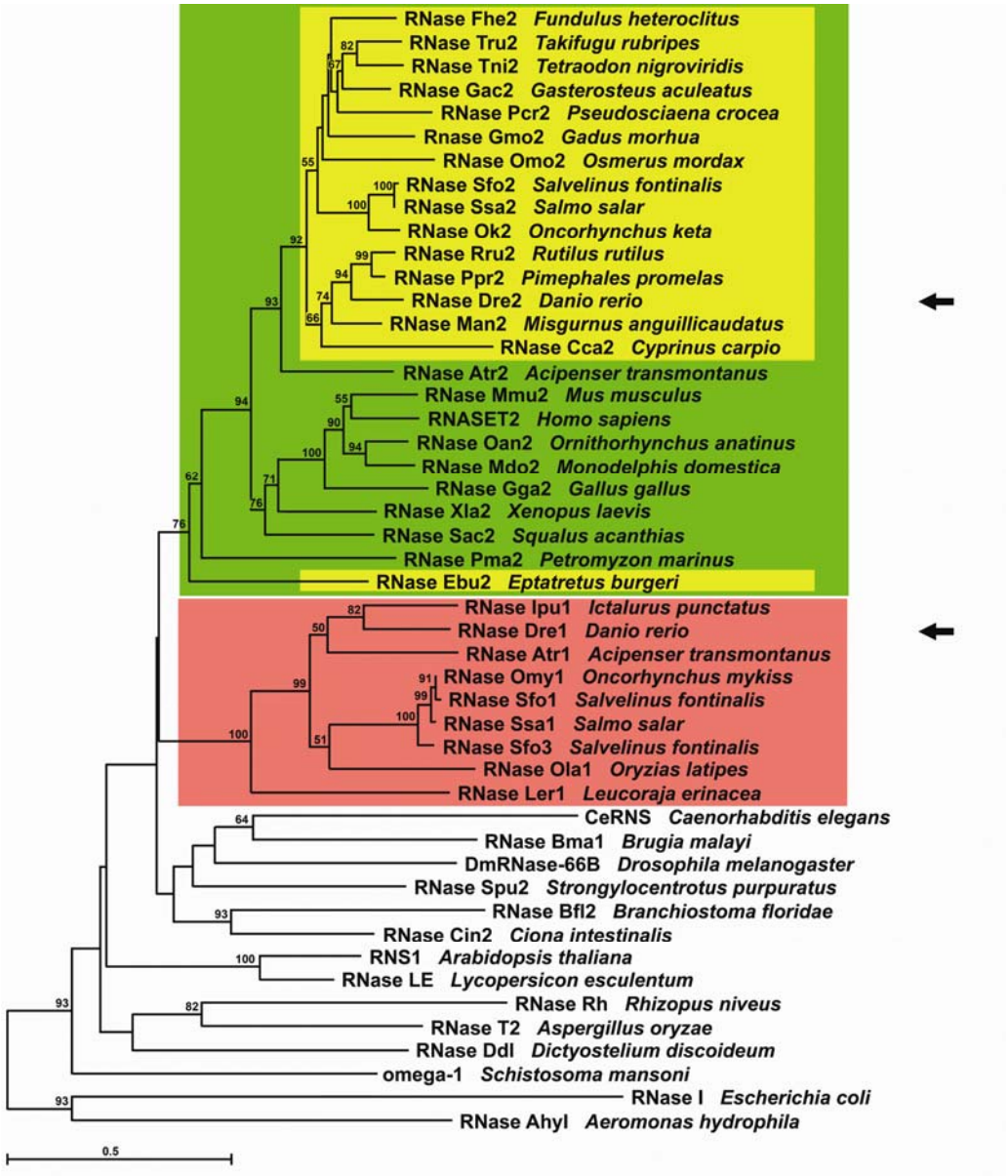

Figure 6 - Phylogenetic relationships of fish RNase T2 proteins, other animal RNase T2 proteins and bacterial, fungal and plant RNase T2s

Unrooted tree was obtained by the Neighbor-Joining method using only conserved regions. Bootstrap percentages (for 1,000 replications) greater than 50 are shown on interior branches. Color boxes highlight the clades that include fish RNases. Green indicates canonical CAS II, while yellow and red indicate mutations that putatively attenuate RNase activity (see figure 7). RNase Dre1 and RNase Dre2 are indicated with arrows.

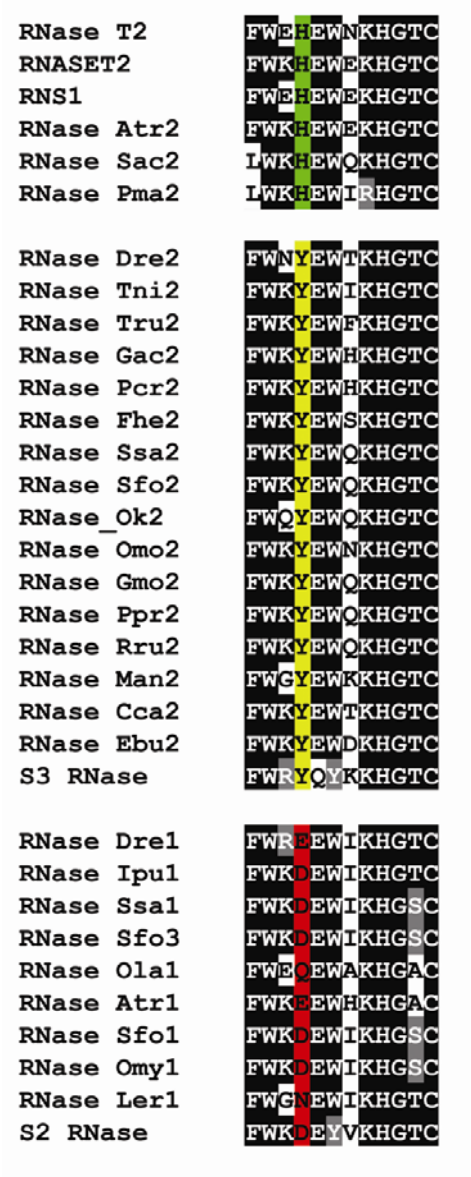

Figure 7 - Mutations in CAS II of fish RNase T2 proteins

The alignment shows the conserved CAS II region characteristic of RNase T2 enzymes. The absolutely conserved His (black box, white font) is part of the catalytic site of the enzyme. A second His residue (green), possibly involved in substrate binding or stabilization of an intermediate in the catalytic reaction, is mutated in most fish RNases. In the RNase Dre2 clade this His is mutated to Tyr (yellow). In the RNase Dre1 clade the His is mutated to a series of polar amino acids (red). Similar mutations are found in some plant S-RNases (S2 RNase and S3 RNase).

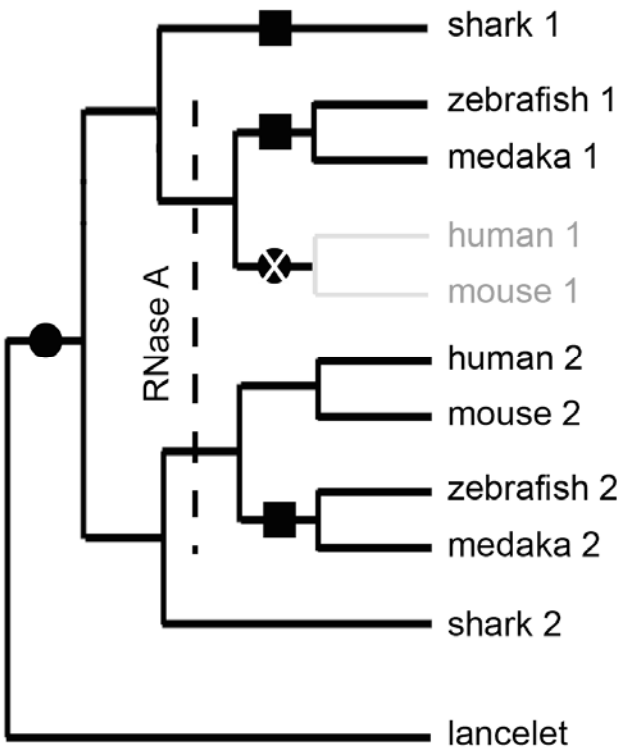

Figure 8 - Hypothetical model of RNase T2 evolution in animals

An ancestral RNase T2 gene present in the last common ancestor of lancelet and higher chordate was duplicated after the separation of these two groups, but before the separation of Chondrichthyes and Teleostomi (black circle). Sometime after this duplication event RNase A genes emerged, most likely after the separation of Chondrichthyes and Teleostomi. The presence of RNase A could have released some selective pressure on RNase T2 genes, allowing the fixation of mutations in the active site conserved region (squares, H/Y/E-D position in CAS II), and the disappearance of one of the genes in tetrapods (black circle with white X).

Supplemental Datasets

RNase Dre1 short mRNA

>RNase_Dre1a
GATATCACAGACTCTCAGAACCCACTGATCAGCATGACCTACAATAAACACATTCTCTGG
GCCTTTACCGCTGCTCTGGCCACAGGTTGGGTCCTTAGTAATGATGAAGGATGCTATTAT
GGGACTGTCATGAAGCACTCATGCAACTGGACTTGTATGCTGCTCACTCTTCAATGGCCT
GGAAGTTTCTGTATAGGTCTTACTAATAAAACGATTTGCAAAATACCACTGACTATTCAA
AACTGGACCATCCATGGCCTATGGCCGATGCATACAGGTCACTGCTGTAACTGTTGGCCA
ATATTTCATTCCCACCTTCAGGAAATCGAGCCAGAACTCACTCAGCTGTGGCCATCTTTA
ATAAAAGGAAAGCATTTCTTCAACTTTTGGAGGGAGGAATGGATTAAACACGGGACGTGT
GCTGGCTGTGACGGGGCCATGGGTTCACCGCTTCTTTACTTCCAGGCTGCAGTCAAGCTT
CGAAAACTCTTTGACATTAATAGCGTCTTGGAAAGCTCTGGAATCAAAGCCTCATGTGAG
GTGTCCTATAAGTATGACGACATAAGCAAGGCCCTGACCTCACTGCTGGGAGACAACTTT
GATTTGCAATGTGTGACTGACAGTAAGGGTCGTGAAGCATGGATCCAGCTGAAGATCCAT
CTTTCGAGGAACCAAACCATTGGATGCCCCACAGAGAAGCAAGACGAAGCATTCTATAAT
TTAGCGTGGTATAAAAGCCCTGGACATCCTTGTCCGAAGAACACCACCATCTTTTTTGTC
CCAATTAACTATGACAATCCTCATGAGCCATGT

RNase Dre1 protein encoded by short mRNA

>RNase_Dre1a
MTYNKHILWAFTAALATGWVLSNDEGCYYGTVMKHSCNWTCMLLTLQWPGSFCIGLTNKT
ICKIPLTIQNWTIHGLWPMHTGHCCNCWPIFHSHLQEIEPELTQLWPSLIKGKHFFNFWR
EEWIKHGTCAGCDGAMGSPLLYFQAAVKLRKLFDINSVLESSGIKASCEVSYKYDDISKA
LTSLLGDNFDLQCVTDSKGREAWIQLKIHLSRNQTIGCPTEKQDEAFYNLAWYKSPGHPC
PKNTTIFFVPINYDNPHEPC

RNase Dre1 long mRNA

>RNase_Dre1b
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TAGCTATATAACCTCTGTTTAAATGCATATTTACTGTAACAATCTGTATTGTTTTAGATA
AACACATTCTCTGGGCCTTTACCGCTGCTCTGGCCACAGGCTGGGTCCTTAGTAATGATG
AAGGATGCTATTATGGGACTGTCATGAAGCACTCATGCAACTGGACTTGTATGCTGCTCA
CTCTTCAATGGCCTGGAAGTTTCTGTATAGGTCTTACTAATAAAACGATTTGCAAAATAC
CACTGACTATTCAAAACTGGACCATCCATGGCCTATGGCCGATGCATACAGGTCACTGCT
GTAACTGTTGGCCAATATTTCATTCCCACCTTCAGGAAATCGAGCCAGAACTCACTCAGC
TGTGGCCATCTTTAATAAAAGGAAAGCATTTCTTCAACTTTTGGAGGGAGGAATGGATTA
AACACGGGACGTGTGCTGGCTGTGACGGGGCCATGGGTTCACCGCTTCTTTACTTCCAGG
CTGCAGTCAAGCTTCGAAAACTCTTTGACATTAATAGCGTCTTGGAAAGCTCTGGAATCA
AAGCCTCATGTGAGGTGTCCTATAAGTATGACGACATAAGCAAGGCCCTGACCTCACTGC
TGGGAGACAACTTTGATTTGCAATGTGTGACTGACAGTAAGGGTCGTGAAGCATGGATCC
AGCTGAAGATCCATCTTTCGAGGAACCAAACCATTGGATGCCCCACAGAGAAGCAAGACG
AAGCATTCTATAATTTAGCGGCGTATAAAAGCCCTGGACATCCTTGTCCGAAGAACACCA
CCATCTTTTTTGTCCCAATTAACTATGACAATCCTCATGAGCCATGTAAC

RNase Dre1 protein encoded by long mRNA

>RNase_Dre1b

MKHSCNWTCMLLTLQWPGSFCIGLTNKTICKIPLTIQNWTIHGLWPMHTGHCCNCWPIFH
SHLQEIEPELTQLWPSLIKGKHFFNFWREEWIKHGTCAGCDGAMGSPLLYFQAAVKLRKL
FDINSVLESSGIKASCEVSYKYDDISKALTSLLGDNFDLQCVTDSKGREAWIQLKIHLSR
NQTIGCPTEKQDEAFYNLAAYKSPGHPCPKNTTIFFVPINYDNPHEPCN

RNase Dre2 mRNA

>RNase_Dre2
ACAGGCTGTTTGTTACTGACAGGAAAGGAAGTGGCTTTAAAGTGACTCAACCGCACATCA
TTTTTTTAAAGAAAGTCTGGGCAGATCTACTTCAGTCAGAGTTATACTAATAGGACGAAA
GGAGAACTGTAACGTTATGTGAATTGGAGCGGCACTTCAGACAATAACAGATTAACTGTT
TTACTCTACCTGGAAACATGAGATTCATTGCATTTGCTGTCATCTTTAGTGCTGTATATC
TTTGCTCATCAGCCTTTACCCATCCTCGGGGAGAATGGACAAAACTTATACTGACCCAGC
ATTGGCCACAGACATTTTGCAAAATGGAACACTGCAAAACAGATTTCAGCTATTGGACTC
TGCATGGATTATGGCCCAACACTGGTGTAAGGTGCAACACATCTTGGCATTTTAATGCCA
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CTTCCCCAAAATTTTGGAATTATGAATGGACGAAACACGGGACCTGTGCTGCAAAATCAG
AGTCTTTAAACAGTGAACATAAGTACTTTGGCAAAGCTCTCGAACTCTACCACAAGTTTG
ACCTTAACAGTGTTTTGCTGAAGAATCAAATTGTGCCCTCTGAGAAGCATTACACGCTGG
AGGATGTGGAGGAAGCCATTACAAGTGCCTACGGAGTAAAGCCCAAGATCCAGTGTGTCC
ACCCAGGACAGGGAGGCCAGGTTCAAATTTTGGGCCAAATAGAGATCTGTGTTGACAGGG
ATTTCCAACTGATGGGTTGTGAAAAGTCCAGCGAAGACACCTGGAGCAATGACCTCCCCA
CAGTGCCTGTCAGTGGCCAGTCAGGACTCAGCGTGTGTGATCACTCCATGCCAGTCTATT
ACCCACCGGTGCAAGCG

RNase Dre2 protein

>RNase_Dre2
MRFIAFAVIFSAVYLCSSAFTHPRGEWTKLILTQHWPQTFCKMEHCKTDFSYWTLHGLWP
NTGVRCNTSWHFNASLIEDILPEMEKFWPDLLEPSSPKFWNYEWTKHGTCAAKSESLNSE
HKYFGKALELYHKFDLNSVLLKNQIVPSEKHYTLEDVEEAITSAYGVKPKIQCVHPGQGG
QVQILGQIEICVDRDFQLMGCEKSSEDTWSNDLPTVPVSGQSGLSVCDHSMPVYYPPVQA

Supplemental File 1 - mRNA and predicted protein sequences for *RNase Dre1* and *Dre2*.
Sequences were deposited into Genbank under accession numbers FJ460210 andFJ460211 for RNase Dre1 (splicing variants) and FJ460212 for RNase Dre2.

Name	Accession #	species	common name	CASII	source
RNase T2	P10281	*Aspergillus oryzae*	Koji-kin	H	Protein
RNase Rh	P08056	*Rhizopus niveus*		H	Protein
RNase Ddl	XP_640939	*Dictyostelium discoideum*		H	Protein
RNase LE	P80022	*Lycopersicon esculentum*	tomato	H	Protein
RNS1	P42813	*Arabidopsis thaliana*	thale-cress	H	Protein
omega-1	ABB73002	*Schistosoma mansoni*		H	Protein
RNase Ler1	CV222118	*Leucoraja erinacea*	little skate	N	EST
RNase Atr1	DR976433	*Acipenser transmontanus*	American sturgeon	E	EST
RNase Ola1	BJ501340	*Oryzias latipes*	medaka	Q	EST
RNase Dre1	**FJ460211**	***Danio rerio***	**zebrafish**	**E**	**this work**
RNase Ipu1	CV994888	*Ictalurus punctatus*	channel catfish	D	EST
RNase Sfo3	EV387897	*Salvelinus fontinalis*	brook trout	D	EST
RNase Ssa1	DW561904	*Salmo salar*	Atlantic salmon	D	EST
RNase Sfo1	EV393106	*Salvelinus fontinalis*	brook trout	D	EST
RNase Omy1	CA371032	*Oncorhynchus mykiss*	rainbow trout	D	EST
RNase Spu2	XP_780287	*Strongylocentrotus purpuratus*	purple sea urchin	H	Protein
RNase Ebu2	BJ653399	*Eptatretus burgeri*	inshore hagfish	Y	EST
RNase Pma2	CO553054 + CO552166	*Petromyzon marinus*	sea lamprey	H	EST
RNase Atr2	DR976045	*Acipenser transmontanus*	American sturgeon	H	EST
RNase Cca2	EX822236	*Cyprinus carpio*	common carp	Y	EST
RNase Fhe2	DR441730	*Fundulus heteroclitus*	killifish	Y	EST
RNase Omo2	EL528012	*Osmerus mordax*	rainbow smelt	Y	EST
Rnase Gmo2	EX741865	*Gadus morhua*	Atlantic cod	Y	EST
RNase Tni2	CAG05697	*Tetraodon nigroviridis*	spotted green pufferfish	Y	Protein
RNase Tru2	CA330011	*Takifugu rubripes*	fugu	Y	EST
RNase Gac2	DW635779	*Gasterosteus aculeatus*	three-spined stickleback	Y	EST
RNase Pcr2	CX349007	*Pseudosciaena crocea*	large yellow croaker	Y	EST
RNase Ok2	BAB55596	*Oncorhynchus keta*	chum salmon	Y	Protein
RNase Ssa2	EG914237	*Salmo salar*	Atlantic salmon	Y	EST
RNase Sfo2	EV393679	*Salvelinus fontinalis*	brook trout	Y	EST
RNase Man2	BJ820572	*Misgurnus anguillicaudatus*	oriental weatherfish	Y	EST
RNase Dre2	**FJ460212**	***Danio rerio***	**zebrafish**	**Y**	**this work**
RNase Ppr2	DT189242	*Pimephales promelas*	fathead minnow	Y	EST
RNase Rru2	EG548700	*Rutilus rutilus*	roach minnow	Y	EST
RNase Xla2	NP_001086583	*Xenopus laevis*	African clawed frog	H	Protein
RNase Sac2	EG027303	*Squalus acanthias*	Dogfish Shark	H	EST
RNase Gga2	NP_001034580	*Gallus gallus*	chicken	H	Protein
RNASET2	NP_003721	*Homo sapiens*	human	H	Protein
RNase Mmu2	NP_001077407	*Mus musculus*	mouse	H	Protein
RNase Mdo2	XP_001381570	*Monodelphis domestica*	gray short-tailed opossum	H	Protein
RNase Oan2	EY201560	*Ornithorhynchus anatinus*	duck-billed platypus	H	EST
RNase Cin2	BW482808	*Ciona intestinalis*	Sea squirt	H	EST
RNase Bfl2	BW708730	*Branchiostoma floridae*	Florida lancelet (Amphioxus)	H	EST
CeRNS	NP_503370	*Caenorhabditis elegans*	nematode	H	Protein
RNase Bma1	EDP29148	*Brugia malayi*	filarial nematode	H	Protein
DmRNase-66B	CAA52884	*Drosophila melanogaster*	fruit fly	H	Protein
RNase Ahyl	CAA47438	*Aeromonas hydrophila*		H	Protein
RNase I	NP_752630	*Escherichia coli*		Y	Protein

Supplemental File 2 – RNase T2 proteins used for phylogenetic analysis
Table of RNases with accession numbers used for generation of phylogenic analysis of T2 RNases for the Neighbor-Joining tree method.

CHAPTER 5: RNase T2 genes from rice and the evolution of secretory ribonucleases in plants

Modified from a paper submitted to BMC Plant Biology

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Alexander Meyer [1,2], Lex Flagel [4]

Authors' contributions

GCM conceived of the study, performed the bioinformatics and phylogenetic analyses, and drafted the manuscript. MSH carried out the gene expression analyses and cloning of rice cDNAs. AM cloned the petunia cDNA and did some preliminary expression analyses. LF participated in the phylogenetic analysis and reviewed the manuscript. All authors read and approved the final manuscript.

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Abstract

Background

Plant ribonucleases (RNases) belonging to the RNase T2 family are classified into two different subfamilies based on their function. S-RNases are involved in rejection of self-pollen during the establishment of self-incompatibility in three plant families. S-like RNases, on the other hand, are not involved in self-incompatibility, and although gene expression studies point to a role in plant defense and phosphate recycling, their biological roles are less well-understood. There is also a marked difference in our understanding of the evolution of the two subfamilies of RNases. While S-RNases have been subjects of many phylogenetic studies, few have included an extensive analysis of S-like RNases, and genome-wide analyses to determine the number of S-like RNases in fully sequenced plant genomes are missing.

Results

In this work we characterized the eight RNase T2 genes present in the *Oryza sativa* genome; and we also identified RNase T2 genes present in other fully sequenced plant genomes. Using phylogenetic analyses and gene expression studies we characterized two different classes of RNase T2 genes, all part of the S-like RNase subfamily. Class I genes show tissue specificity and are regulated by stress, as previously shown for S-like RNases. Inactivation of RNase activity has occurred repeatedly throughout evolution, including a highly conserved monocot-specific clade of RNase T2 genes in Class I lacking RNase activity. On the other hand, Class II seems to have conserved more ancestral characteristics; and, unlike other S-like RNases, genes in this class are constitutively expressed and conserved in all plant species analyzed.

Conclusions

Our results suggest that upon gene duplication, Class I genes may have undergone subfunctionalization that resulted in active RNase genes, and genes that are still clearly recognized as members of the RNase T2 family but that have lost their RNase activity. Many of these genes are differentially expressed in

response to stress, and we propose that protein characteristics such as the basic nature of some of their domains can have a defense role independent of RNase activity. On the other hand, constitutive expression and phylogenetic conservation suggest that Class II S-like RNases may have a housekeeping role.

Background

Ribonucleases (RNases) belonging to the RNase T2 family are acidic endoribonucleases without base specificity that are targeted to the secretory pathway. These RNases have molecular masses of the protein moiety (some enzymes of this family are glycosylated) between 20-25 kDa, and are typified by RNase T2, an extracellular RNase from *Aspergillus oryzae* [1]. The discovery that some RNase T2 family members (called S-RNases) are involved in gametophytic self-incompatibility [2] to detailed analyses of this type of enzymes in plants [3]. In addition to S-RNases, present in the Solanaceae, Scrophulariaceae, and Rosaceae families, plants possess other RNase T2 enzymes, known as S-like RNases, which are not involved in self-incompatibility [4]. S-like RNases have been found in all plant species, but their evolution and biological role are less well understood.

Members of the RNase T2 family are in fact present in the genome of almost all organisms, including bacteria, viruses, fungi, animals and plants [5] [6] [7], with the exception of some bacteria [8] and trypanosomes (G. MacIntosh, unpublished). This almost absolute conservation during the evolution of eukaryotes suggests an important function for this family of RNases [7].

Extensive analysis of gene expression in plants suggests that S-like RNases are involved in phosphate recycling during senescence and are up-regulated during periods of inorganic phosphate (Pi)-starvation. Expression of two tomato RNases, *RNase LE* and *RNase LX*, is induced when cultivated tomato cells or seedlings are grown in Pi-deficient media [9]. Two RNase T2 genes from Arabidopsis, *RNS1* and *RNS2*, are also induced by Pi-starvation [10] [11]. In addition, expression of *RNS2* and *RNase LX* increases during senescence [10] [12], and *ZRNaseI* from Zinnia is expressed in the late stage of *in vitro* tracheary element differentiation [13], indicating that the corresponding proteins could be involved in recycling of phosphate during processes involving cell death. Moreover, antisense suppression of *RNase LX* expression results in delayed senescence and leaf abscission, suggesting that RNase LX may not only recycle phosphate during senescence and abscission, but could also participate in the control of these processes [14].

S-like RNases also participate in defense responses. Tobacco *RNase NE* expression is induced in response to *Phytophthora parasitica*, and purified RNase NE can inhibit hyphal elongation of this pathogen [15]. Other *Nicotiana* genes, *RNase NW* and *RNase Rk1*, are induced in response to tobacco mosaic virus and cucumber mosaic virus infections, respectively [16] [17]. Mechanical wounding also induces expression of several S-like RNases, including *RNS1*, *RNase NW*, *ZnRNaseII*, *RNase LE* and *RNase Nk1* [18] [19] [13] [20]. Although the role of S-like RNases during wounding is not clear, it has been proposed that they could be antimicrobial enzymes, or participate in phosphate remobilization during the healing process [18] [17].

S-like RNases are also found in monocotyledonous plants, although they have been studied to a lesser extent. Two genes belonging to the RNase T2 family have been described in rice. The first, *RNase Os*, encodes an active enzyme that was purified from rice bran [21]. A barley enzyme, GAR-RNase, also accumulates in the aleurone layer, and *GAR-RNase* expression is induced by gibberellins and repressed by abscisic acid [22]. It was proposed that this enzyme may contribute to digestion of RNA in the dead starchy endosperm cells and thereby may function to help mobilize Pi for the developing embryo [22]. A second rice gene belonging to this family was identified by a proteomic analysis of rice leaves undergoing drought stress [23]. The most highly induced protein in that experiment, RNase DIS, has homology to RNase T2 but lacks the conserved residues in the active site of the enzyme. The same gene, though renamed *OsRRP* [24], was reported to be expressed preferentially in stems and to be down-regulated in an increased tillering dwarf mutant. The biological role of this non-functional RNase is unknown [24]. Similar non-functional S-like RNase genes have been found in wheat [25] and barley [26].

In spite of extensive phylogenetic analyses of S-RNases in several plant families [27] [28] [29], a genome-wide survey of the entire RNase T2 enzyme family has not been done for any plant species. Since S-like RNases from monocots have been sparsely studied, and since the full sequence of the rice genome is available [30], we set out to characterize the RNase T2 family in this species. We identified eight RNase T2 genes in the rice genome. Using phylogenetic analysis and gene expression studies we characterize two different

classes of RNase T2 genes, all part of the S-like RNase subfamily. Class I genes show tissue specificity and are regulated by stress, as expected for S-like RNases. Among this class, inactivation of RNase activity has occurred repeatedly throughout evolution, including a highly conserved monocot-specific class of RNase T2 genes lacking RNase activity. On the other hand, Class II seems to have conserved more ancestral characteristics; and, unlike other S-like RNases, genes in this class are constitutively expressed and conserved in all plant species analyzed. These characteristics suggest that Class II S-like RNases may have a housekeeping role.

Results

The rice genome contains eight RNase T2 genes

A survey of the two available annotation databases for rice, *Oryza sativa*, revealed that several RNase T2 genes are present in its genome. Searches in the Michigan State University (MSU) Rice Genome Annotation Database [31], which contains sequence information for the subspecies *japonica* c.v. Nipponbare, returned eight gene models with homology to RNase T2 proteins. In contrast, searches in the most current version of the Rice Annotation Project Database (RAP-DB, [32]), also based on the sequence of Nipponbare, produced only six gene models belonging to this family. These six genes corresponded to six of the eight genes identified in the MSU database; a search in previous versions of RAP-DB using the sequences of the two genes only present in the MSU database showed that these genes were also present in older RAP-DB assemblies.

Additional searches using BLASTP and BLASTN, either on gene models or the complete genome assembly deposited in Phytozome (www.phytozome.net), failed to identify any other sequences with homology to the RNase T2 family. Based on analysis of EST sequences (not shown) and our own gene expression experiments (see below) we are confident that the eight genes correspond to expressed proteins belonging to this ribonuclease family. We named these genes *OsRNS1-8*, following the nomenclature used for the

RNase T2 genes found in *Arabidopsis thaliana* [33]. The corresponding IDs from the two rice annotation databases are provided in Table 1.

We performed an analysis of the intron structure of the genes in the rice RNase T2 family (Figure 1). Four genes, *OsRNS1*, *OsRNS3*, *OsRNS7* and *OsRNS8*, contain only one intron within the coding region, although the *OsRNS7* and *OsRNS8* gene models do not have EST support. *OsRNS3* has an additional intron in the 3' UTR. On the other hand, *OsRNS2*, *OsRNS4*, *OsRNS5* and *OsRNS6* have multiple introns in the coding region. *OsRNS4*, *OsRNS5* contain three and four introns respectively; while *OsRNS2* and *OsRNS6* have seven and nine.

The proteins encoded by these genes (Figures 1 and 2) have predicted molecular weights between 24 and 32 kDa, similar to other RNases in the family [6]. All are predicted to have signal peptides that would target these proteins to the secretory pathway (Figure 2), as is the norm among RNase T2 proteins. The predicted subcellular localization for the eight rice proteins is shown in Figure 1. Several rice RNases have acidic pIs, as described for most S-like enzymes; however OsRNS2 has a near neutral pI, and OsRNS5 and OsRNS6 have basic pIs. Most S-like RNases have been found to have acid pIs while basic pIs are characteristic of S-RNases, although some basic S-like RNases have been found ([34] and references therein).

The eight rice genes encode proteins with homology to RNase T2 proteins, and are more similar to S-like RNases than S-RNase (not shown, but see below); however, the level of conservation of amino acid residues that are important for ribonuclease activity and structure [5] varies among them (Figure 2). Five proteins (OsRNS2, OsRNS6, OsRNS8, OsRNS1, OsRNS3) contain the two histidines that are present in the conserved active site (CAS) I and II motifs, and are essential for ribonuclease activity [5]. OsRNS3 corresponds to the previously described RNase Os, an active RNase purified from rice bran [21]. On the other hand, OsRNS4 and OsRNS5 have lost most conserved residues in the two CAS. These changes are *bona fide* mutations, not the result of sequencing errors, as the same sequences can be found in the genome of *japonica* and *indica*, and in many EST collections for both subspecies (not shown). As a consequence, these

two proteins have clearly lost their ribonuclease activity, as previously described for RNase DIS [23] (a.k.a. OsRPP [24]), which corresponds to OsRNS4.

OsRNS7 also has a mutation in the first active site His. In this protein the amino acid in this position has changed to Arg. This could be seen as a conservative change; however CalsepRRP, the major protein of resting rhizomes of *Calystegia sepium* and also a member of the RNase T2 family, has a substitution to Lys in the same position and is devoid of any ribonuclease activity [35]. Thus, it is likely that OsRNS7 is also an inactive protein. However, since no ESTs were found for this gene, it would be possible that *OsRNS7* represents a pseudogene. To test this idea, we obtained rice mRNA and amplified a cDNA corresponding to *OsRNS7*. This is the first proof of expression of *OsRNS7*, and it indicates that this gene is not a pseudogene. Sequencing of this cDNA also confirmed the change in CAS I. RT-PCR analysis was also used to confirm expression of *OsRNS8*, the other rice gene for which proof of expression was missing. Both sequences have been deposited in GenBank under accession numbers GQ507488 and GQ507489, respectively.

Finally, although OsRNS6 has both active site His residues conserved, this protein has a substitution in the first Lys of CAS II. This is also a *bona fide* change since all *japonica* ESTs corresponding to this gene have the same mutation, and the same sequence can be found in the *indica* genome (GenBank accession number EEC71956). Biochemical characterization of *Rhizopus niveous* mutant RNases indicates that this residue is important for activation of the enzyme [5]. Changes in this position can have different degrees of effect on RNase activity; a K108T substitution resulted in a protein with 20-fold less activity than WT RNase Rh [36]. Thus, OsRNS6 could be an attenuated RNase.

Structural residues, like Cys residues involved in disulfide bridges that are important for proper folding [5], also show different levels of conservation. Interestingly the Cys residue in CAS II is absolutely conserved, even though the residues involved in activity are not.

Tissue specificity and stress regulation of OsRNSs expression

As a first step toward characterizing the RNase T2 family in rice we analyzed the expression of the eight genes in different organs, and took

advantage of public data from expression databases to identify biotic and abiotic stresses that regulate the expression of rice S-like RNase genes. We obtained root, leaf, stem, and inflorescence tissue from adult rice plants, prepared RNA and tested for the presence of RNase T2 transcripts by RT-PCR (Figure 3). Expression of *OsRNS2*, *OsRNS3*, *OsRNS4*, and *OsRNS8* was detected in all organs. Although our RT-PCR assay is only semi-quantitative, it is evident that even though *OsRNS4* is present in all tissues tested, its expression is stronger in stems. *OsRNS5*, *OsRNS6* and *OsRNS7* show stronger organ specificity: *OsRNS5* is only expressed in stem and inflorescences, *OsRNS6* is expressed in all tissues except leaves, and *OsRNS1* and *OsRNS7* is only detected, in roots.

Analysis of public expression data (microarrays and MPSS - Massively Parallel Signature Sequencing) confirmed the tissue-specificity observed in our experiments (Table 2). Moreover, this analysis indicated that many of the *OsRNS* genes are regulated by biotic and abiotic stress (Table 2). We were particularly interested in regulation by Pi-starvation, since one of the functions proposed for this family of enzymes is phosphate remobilization in response to limitations in this nutrient's availability. Three genes showed elevated expression in response to low Pi. *OsRNS8* was induced in leaf and root tissues. Interestingly, the root-specific *OsRNS7* and *OsRNS5* are also induced in roots in response to Pi-starvation, even though these proteins lack the amino acid residues necessary for ribonuclease activity.

Another proposed function for this family of RNases is a role in plant defense against pests. Consistent with this idea, *OsRNS4* and *OsRNS5* showed increased expression in response to insect attacks (beet armyworm, *Spodoptera exigua*, and water weevil, *Lissorhoptrus oryzophilus*) and wounding. Expression of *OsRNS4* and *OsRNS5* was also differentially increased in response to the bacterium *Xanthomonas oryzae*, while *OsRNS7* was differentially expressed in response to the fungus *Magnaporthe grisea* (Table 2). Remarkably, neither of these proteins seems to be an active RNase, suggesting that the defense role for these proteins should be independent of the enzymatic activity.

Several *OsRNS* genes are also induced by abiotic stresses, with the most common regulator being high salt concentration. *OsRNS3, 4* and *5* are induced in response to this stress condition. *OsRNS3* expression is also up-regulated by

drought and cold, while *OsRNS8* expression is up-regulated only in response to cold.

Phylogenetic analysis of plant RNase T2 proteins

To gain insights into the evolution of the S-like RNase family in plants, we performed a phylogenetic analysis including all the proteins belonging to this family present in several fully sequenced plant genomes. Our study comprised genes from rice, Arabidopsis (*Arabidopsis thaliana*)[37], black cottonwood (*Populus trichocarpa*)[38], sorghum (*Sorghum bicolor*)[39], and soybean (*Glycine max*). We added several known plant S-like RNases from eudicots, monocots, moss, and algae. We also included protein sequences derived from EST collections or cDNA collections, although this is not an exhaustive list of plant S-like RNases.

Searches in the fully sequenced genomes were performed using the genome assemblies deposited in Phytozome (www.phytozome.net), using TBLASTN. These analyses resulted in the identification of the five already known RNase T2 genes in Arabidopsis [33] [27], five genes also in cottonwood (*PtrRNS1-5*; but see below), twelve genes in soybean (*GmaRNS1-12*), and six genes in sorghum (*SbRNS1-6*). We also identified a partial RNase T2 sequence in the cottonwood chromosome LG X with high degree of similarity to *PtrRNS1*, a partial sequence similar to *PtrRNS4* in a scaffold not included in the assembled chromosomes, and a sequence with similarity to RNase T2 proteins in chromosome LG XII that has several stop codons in the putative coding region, suggesting that it is a pseudogene. These sequences were not included in our analysis due to the lack of gene models or ESTs supporting their expression.

In total, 78 protein sequences were analyzed using a Bayesian phylogenetic approach to create a tree of plant S-like RNases (Figure 4). A full list of the proteins with the corresponding accession number and species are shown in Additional file 1. The Bayesian tree presented a well defined clade for only a small portion of the sequences, but the rest of the tree had less definition. Thus, a Neighbor-Joining tree was also made (Figure 5 and Additional file 2) to see if a different method could resolve this portion of the tree. However, the results from both approaches were similar. The trees allowed us to make several

inferences on the evolution of the RNase T2 family in plants. First, our analysis agrees with previous phylogenetic studies that sorted plant RNase T2 proteins into three classes [27] [28] [40]; Class I and Class II correspond to S-like RNases, while Class III corresponds to S-RNases. Though our analysis did not include S-RNases, we did identify two main clusters for S-like RNases corresponding to Classes I and II (we use the class names as defined in [27]). Furthermore, the presence of only one RNase T2 gene in fully sequenced genomes of green algae [41] [42] suggests that the gene duplication that gave rise to the two classes of RNases in the S-like subfamily occurred after the split of Chlorophyta from the main Viridiplantae stem.

Only RNases belonging to Class I could be found in the moss *Physcomitrella patens* and in the spikemoss *Selaginella moellendorffii*, both of which have fully sequenced genomes [43] [44]. The earliest plant in which both classes of RNases were found is a conifer (*Picea glauca*). This could mean that the duplication that originated Class I and Class II happened after the separation of Euphyllophyta and Lycopodiophyta, and before the separation of Gymnosperms and Angiosperms. However, it seems that Class II conserved more ancestral characteristics because some algal RNases and the only sequence obtained from a Marchantiophyta (*Marchantia polymorpha*) are included in the Class II clade. Thus, an alternative hypothesis could be that the gene duplication occurred early in the Embryophyte branch, but Bryophytes and Lycopodiophyta could have lost the gene corresponding to Class II after the split from the main land plant branch.

As mentioned, part of the Bayesian tree is well resolved. The resulting strongly supported clade corresponds to the Class II S-like RNases. This clade includes at least one gene from each of the fully sequenced higher plant genomes, and in the cases in which more than one gene for this class was identified (rice and soybean) the gene duplications have likely occurred recently, as they form monophyletic groups. Moreover, in general, the associations among Class II RNases follow established taxonomic relationships.

In contrast, Class I S-like RNases form a clade with little structure, with several proteins from each genome that do not form species-specific clades, indicating that the gene duplication events that originated these RNases predate

speciation. Then, diversification of Class I RNases resulted from differential retention and expansion in different lineages by gene sorting, a process characterized by rapid gene duplication and deactivation occurring differentially among lineages [45]. For example, Arabidopsis RNS4 and soybean GmaRNS9 form a small clade with strong support. This indicates that the gene duplication that gave rise to these genes occurred before the separation between Eurosids I (which includes soybean) and Eurosids II (which includes Arabidopsis). In further support of gene sorting, no ortholog to the aforementioned clade could be found in cottonwood, which also belongs to the Eurosids I, suggesting that in this species this gene was lost. Similarly, gene duplication and loss events can be evoked to explain the clade containing Arabidopsis RNS3 and RNS5 among other proteins.

One major source of incongruence in phylogenetic reconstructions is homoplasy [46]. To establish its effect on our analysis of plant RNS genes, we performed a parsimony analysis (not shown) of the same sequences, which produced highly similar tree topologies to the Bayesian and Neighbor-Joining methods, and used this tree to calculate the homoplasy index (HI). The HI for the whole tree was unusually high (0.5460). However, when we partitioned the data set into only Class I and II groups the sequences in the Class II clade had an HI of only 0.1588, whereas the Class I clade had an HI of 0.3807, indicating that Class I is primarily responsible for the high HI index of the tree containing the whole set of sequences.

Among Class I S-like RNases another cluster with strong support can be identified. This clade is monocot-specific and includes proteins that have lost their ribonuclease activity due to mutations in CAS I and/or CAS II (Figure 6). This clade includes two of the three inactive RNSs from rice, OsRNS4 and OsRNS5. Barley and sorghum also have the two homologs of OSRNS4 and OsRNS5. Thus, in this case the gene duplication that originated the clade may have occurred in an ancestral grass genome, before the whole genome duplication that gave rise to the modern genomes [47]. Although these genes have mutations that likely result in no RNase activity, they are clearly not pseudogenes, since high levels of expression were detected for *OsRNS4* and *OsRNS5* in different

tissues (Figure 3), and high levels of OsRNS4 protein accumulate in response to drought [23].

Interestingly, inactivation of the CAS I and CAS II RNase domains happened more than once during the evolution of the S-like RNases. Another rice protein, OsRNS7, has a mutation in CAS I that likely inhibits RNase activity. This protein does not cluster with the monocot-specific clade. Inactive RNases are also present in eudicot genomes (Figure 6). CalsepRRP, the major protein of resting rhizomes of *Calystegia sepium*, is an inactive RNase [35] that belongs to the Class II S-like RNases; in addition, we identified three soybean genes (*GmaRNS3*, *GmaRNS4*, and *GmaRNS5*) that encode for inactive RNases belonging to Class I.

Class II RNases are highly conserved and expressed constitutively

The Class II clade contains one homolog from each fully sequenced seed plant genome, with the exception of soybean and rice, which have two proteins in the clade. Both grasses and plants of the genus *Glycine* have undergone ancestral whole genome duplication events [47] [48], which may explain the second gene belonging to Class II.

Analysis of gene expression indicates that *OsRNS2* is expressed constitutively in all tissues and all developmental stages (Figure 3 and Table 2, and data not shown). The conservation of these genes could extend also to their expression since Arabidopsis *RNS2*, the other gene for which expression analyses are available, is also expressed constitutively in all tissues and developmental stages ([10]; A. Meyer and G.C. MacIntosh, unpublished). This could indicate that the Class II S-like RNases have an important function that has preserved these enzymes throughout evolution. If this is the case, we would expect that other members of this clade should have similar expression patterns, and that plants for which a Class II S-like RNases has not been found are likely to have a homolog in their genome.

To test these hypotheses we prepared RNA from different tissues from tomato, and analyzed expression of *RNaseLER*, the tomato Class II RNase (Figure 7 A). We observed that, as expected, *RNaseLER* is expressed constitutively in root, stem, flower, and leaf tissues. We also used primers

designed to amplify Solanaceae Class II RNases to search for a Class II sequence in another plant of the family, *Petunia hybrida*, for which no Class II S-like RNase is known. RT-PCR analysis resulted in the amplification of a cDNA fragment corresponding to a Class II S-like RNase as expected (Figure 7 A, B). Gene expression analysis indicated that this gene, which we called *RNase Phy2*, is also expressed in root, stem, flower, and leaf tissues (Figure 7A). We sequenced this cDNA fragment; BLAST analyses showed that the encoded protein is highly similar to tobacco and tomato RNases from Class II (Figure 7 B and not shown). The *RNase Phy2* sequence has been deposited in GenBank under the accession number GQ507487. These results suggest that in fact Class II RNases are conserved and that they are expressed constitutively in all tissues of the plant.

Discussion

Ribonucleases belonging to the RNase T2 family are highly conserved among all organisms. While their enzymatic properties are well understood, their biological roles are still not clear. S-like RNases, a subfamily of the RNase T2 family, are present in the genomes of all plants studied thus far; however, there has not been a genome-wide analysis of this gene family prior to our study. In this work we analyzed the rice genome and found eight genes belonging to the S-like RNase family. We detected expression of every one of these genes in at least one rice tissue, indicating that they are all functional. We also analyzed several other plant genomes, and found that the number of S-like RNases in each genome is variable, from five genes in Arabidopsis to 12 in soybean. This finding suggests a great degree of diversification for the S-like RNase family in plants.

Rice S-like RNases also show diverse expression patterns. *OsRNS2* is highly expressed in all tissues (Figure 3 and Table 2). Also, it does not appear that this gene is regulated by stress conditions, either biotic or abiotic. The other genes are expressed at different intensities, have tissue specificity, and are regulated by abiotic and/or biotic stress. Phylogenetic analyses indicate that S-like RNases can be clustered in two classes, Class I and Class II (Figures 5 and

6, [27] [28] [40]). OsRNS2 clusters with Class II. This finding is also supported by the intron-exon structure of the genes (Figure 1). Class II RNases have seven or more introns [27], as is the case for *OsRNS2* and *OsRNS6*, the two Class II genes from rice. On the other hand, Class I genes have normally between one and three introns, as was found for *OsRNS1*, *OsRNS3*, *OsRNS4*, *OsRNS7* and *OsRNS8*. The last gene, *OsRNS5*, has an extra intron (four), but phylogenetic analysis clearly positions this RNase in the Class I category.

RNases in Class I form a cluster with little structure. The diversification of Class I RNases seems be the result of gene sorting [45]. This idea is also supported by the high homoplasy observed for these RNases. Similar high levels of homoplasy have been shown to be the result of either positive selection or purifying selection [49]. Gene sorting is a common feature of several host-defense gene families, including the vertebrate-specific RNase A family [50]. Accordingly, expression of several Class I rice genes is regulated by pathogens and pests, which suggests that the encoded proteins can have a role in defense. Other S-like RNases have also been implicated in defense. For example, expression of *RNase NE*, a Class I S-like RNase from *Nicotiana tabacum,* is induced in response to *Phytophthora parasitica* [51]. Furthermore, purified RNase NE has antimicrobial activity, and can inhibit hyphal elongation of *P. parasitica* [15]. It was also reported that a highly similar gene from *N. tabacum*, *RNase Nk1*, is induced by cucumber mosaic virus infections [17]; and expression of *RNase NW* is induced in *Nicotiana glutinosa* plants infected with tobacco mosaic virus [16]. Many S-like RNases are also induced by wounding and insect infestations [13] [18] [19] [20] [52]. Thus, the evolutionary pattern observed for Class I RNases could reflect a high rate of gene turnover that is consistent with the role of the gene family in defending against the different pathogens and pests encountered by each plant species. A similar explanation has been proposed for the evolution of the RNase A family in animals [45] [50].

It is important to note that the rice S-like RNases associated with defense responses are all proteins that have lost their RNase activity due to changes in the conserved active sites (OsRNS4, OsRNS5 and OsRNS7). This seemingly unusual finding also has a parallel in the RNase A family. Several members of the RNase A family have antimicrobial properties. Eosinophil associated RNases

have antiviral (RNase 2 and RNase 3 in humans) and antibacterial (RNase 3) functions, and angiogenin and RNase 7 have antibacterial and antifungal activities (reviewed in [53]). However, their ribonuclease activity is not necessary for this antimicrobial role [54] [55]. It has been proposed that their antimicrobial activity is due to their membrane destabilizing properties. A similar mechanism could be envisioned for inactive S-like RNases, like OsRNS4, OsRNS5 and OsRNS7, which are produced in response to pathogen attacks.

Several rice Class I S-like RNases are also induced by abiotic stresses that limit water availability, like drought and high salinity. Comparable regulation has been observed for Arabidopsis RNS1. This RNase is regulated by abscisic acid [56], the main hormone involved in regulation of water stress in plants [57], and is induced in response to high salinity (M. Hillwig and GC MacIntosh, unpublished). Again, it is not clear whether RNase activity is important for a role in this abiotic stress response, since OsRNS4 and OsRNS5, two inactive RNases, are highly induced in response to water stress (Table 2 and [23]).

The presence of inactive RNases could be the result of subfunctionalization or neofunctionalization of the genes resulting from a gene duplication event [58] [59]. Although more research is needed to determine which case applies to these S-like RNases, some evidence from yeast and humans points to subfunctionalization. Only one RNase T2 gene is present in the yeast genome [60]. Thompson and Parker [61] demonstrated that this protein is responsible for the cleavage of tRNAs in response to oxidative stress, and that it can modulate cell survival in response to this stress condition. While tRNA cleavage is dependent on ribonuclease activity, the modulation of cell fate is independent of RNA catalysis. Similarly, human RNASET2, an active RNase, is a tumor suppressor that functions in a manner independent of its ribonuclease activity [62] [63]. Thus, in these cases it is evident that two different functions reside in the same polypeptide. After gene duplication in plants, it is possible that the two functions present in the original gene were individually conserved in each of the duplicates.

Class II S-like RNases, on the other hand, seem to be constitutively expressed; and they are conserved in all the plant genomes analyzed. These RNases seem to have conserved more ancestral characteristics, since they

cluster with lower plant RNases. Evolution of this clade is similar to that observed for the RNase T2 family in animals [7]. Characterization of zebrafish RNase T2 homologs indicated that animal RNase T2 genes are also expressed constitutively; and based on gene expression and phylogenetic analyses, we proposed that animal RNaste T2 proteins have a housekeeping role [7]. The conservation of Class II S-like RNases, expression in all tissues analyzed, and ancestral placement in the phylogenetic trees suggest that these enzymes could also have a housekeeping role in plants. This hypothesis is further supported by similarities in subcellular localization between Class II S-like RNases and animal RNase T2 enzymes, such as RNASET2, which has been shown to localize in the lysosome in human cells [64]. Similarly, Arabidopsis RNS2 is an intracellular enzyme that may be located in the vacuole, the ER, or both ([65] [66], A Meyers and GC MacIntosh, unpublished); and the two Class II RNases in rice are predicted to have an intracellular localization (Figure 1).

Conclusions

The rice genome contains eight genes belonging to the S-like RNase family. These genes belong to two different phylogenetic classes. Class I S-like RNases seem to have undergone diversification through differential fixation and loss of genes after gene duplication events. Many genes in this class are regulated by biotic and abiotic stresses, and are tissue- or organ-specific. They seem to be part of a defense response against a variety of pests, although this role may not be dependent on catalytic activity, since several of the genes induced by pests have mutations that should result in proteins with no RNase activity.

On the other hand, Class II S-like RNases are conserved in all plant genomes analyzed, and the genes are expressed constitutively. Similarities with animal RNase T2 proteins, in terms of evolution, gene expression and protein localization suggest that Class II S-like RNases could be performing a housekeeping function similar to that proposed for animal RNase T2 proteins.

Materials and Methods

Database searches, sequences identification, and protein sequence analyses.

Identification of rice RNase T2 genes was done by BLAST searches [67] using the Arabidopsis RNS1 and RNS2 protein sequences as query in the *Oryza sativa* genome available at Phytozome (http://www.phytozome.net/rice.php), which corresponds to TIGR Release 5 of the annotation of the genome of the japonica subspecies of *O. Sativa*, and the annotated genome assemblies at the MSU Rice Genome Annotation Database [31], and the Rice Annotation Project Database (RAP-DB, [32]). Then the rice sequences obtained were used to re-search the rice genome. Sequences from soybean (*Glycine max*), sorghum (*Sorghum bicolor*), black cottonwood (*Populus trichocarpa*), spikemoss (*Selaginella moellendorffii*), the moss *Physcomitrella patens*, and the green alga *Chlamydomonas reinhardtii* were obtained by BLAST searches of the current version of their respective genomes, as of June 2009, accessed through Phytozome. Genome searches of the green algae *Volvox carteri* and *Ostreococcus tauri* were performed using the assemblies deposited at the Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/); and the *Arabidopsis thaliana* genome was searched using the TAIR9 genome release from the Arabidopsis Information Resource [37]. Additional sequences were obtained by BLASTP searches of the non-redundant protein database and TBLASTN searches of the non-human/non-mouse EST database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Parameters for the predicted protein sequences were obtained using the ProtParam tool at ExPASy [68]. Prediction of signal peptides and subcellular localization was carried out using PSORT [69], WoLF PSORT [70], and SignalP and TargetP [71]. Phosphate-starvation microarray data [72] were obtained from Dr. Huixia Shou (Zhejiang University); other microarray data were obtained using Genevestigator [73]. Massively parallel signature sequencing data (MPSS) were obtained using the Rice MPSS database [74].

Plant material and analysis of gene expression

Rice plants from the subspecies *japonica* c.v. Nipponbare were grown in growth chamber with 12 h photoperiod at 30°C at day time and 28°C at night. Tissue samples were collected at day 75 after sowing. RNA extraction was performed using TRI RNA extraction buffer (Ambion) following manufacturer's instructions. All rice material was provided by Dr. Bing Yang (Iowa State University). *Petunia hybrida* plants were obtained from a local market and maintained in the greenhouse, tissue samples were collected from adult plants about 3-month old. Tomato plants (*Solanum lycopersicum* c.v. Early Girl Hybrid) were grown in growth chamber with 16 h photoperiod at 21°C until they reached maturity. Petunia and tomato RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), RNA was then treated with DNase to remove genomic DNA contamination, and cDNA was synthesized using the i-Script Select Kit (Bio-Rad) following manufacturer's protocols. PCR was performed using GoTaq Green MasterMix (Promega), and the number of cycles was optimized for each primer pair. Primers used are shown in Additional file 3. PCR products were analyzed in 1% agarose gels. For cloning, PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), and the purified fragments were cloned into the pGEM®-T Easy Vector (Promega). Clones were sequenced at the DNA Sequencing Facility at Iowa State University. Partial cDNA sequences for *OsRNS7*, *OsRNS8* and *RNase Phy2* were deposited in GenBank under the accession numbers GQ507488, GQ507489, and GQ507487, respectively. All experiments were repeated at least 3 times, with two independent biological replicates. Representative samples were chosen for each figure.

Phylogenetic analyses

Protein sequences were aligned using the CLC bio software package, followed by manual adjustments. PAUP 4.0 software [75] was used for Neighbor-Joining (1,000 bootstrap replications) and parsimony analyses. Mr Bayes [76] was used for Bayesian tree estimation, using a 1,000,000 generation run length and allowing the program to optimize between 9 different amino acid substitution matrices (using the "prset aamodelpr=mixed" command), while leaving all other

parameters at their default settings.

Acknowledgements

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FIGURES

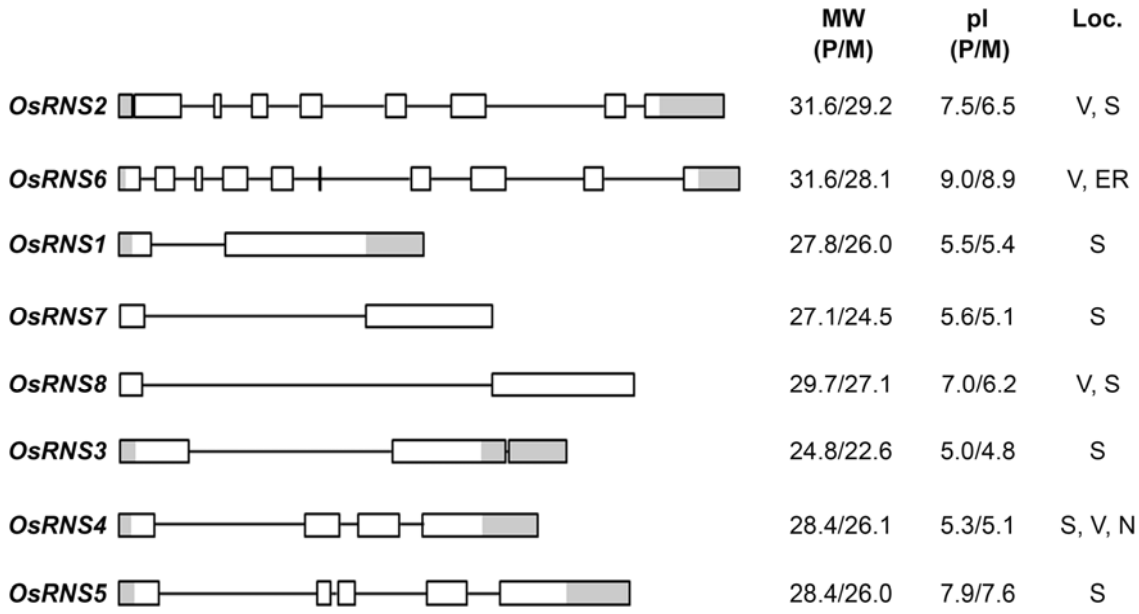


Figure 1 - Structure of rice RNase T2 genes and properties of the encoded proteins

Open reading frames are shown by open boxes, and untranslated regions are shown by gray boxes. Introns are shown as lines. Predicted molecular weight (MW) and isoelectric point (pI) of the pre-proteins (P) and mature proteins (M) are indicated. Signal peptides were predicted using Signal P. Predicted subcellular localizations of the mature peptides are also indicated. V, vacuole; S, secreted (apoplast); ER, endoplasmic reticulum; N, nucleus.

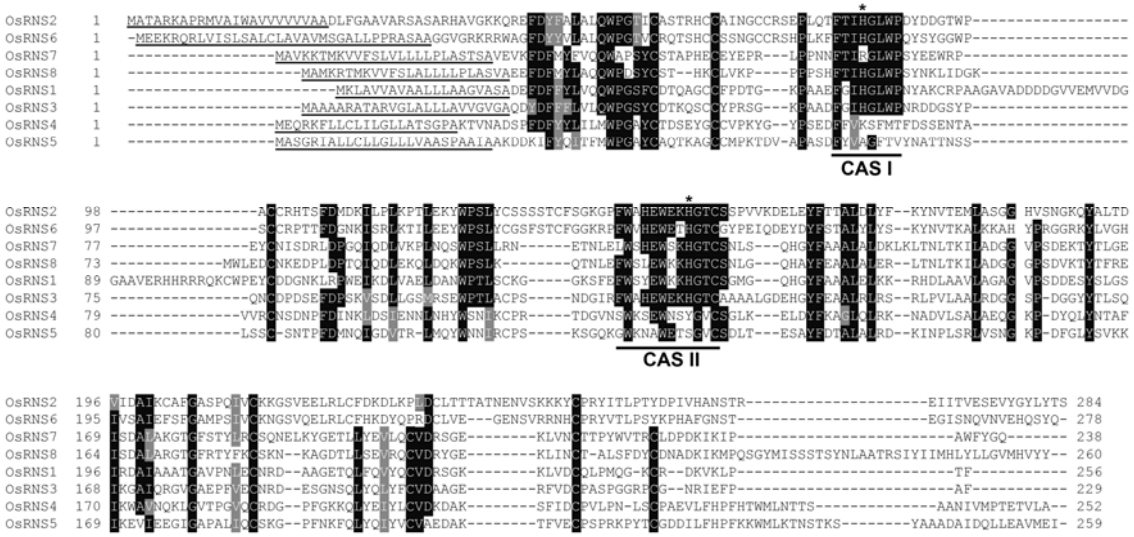


Figure 2 - Protein sequence alignment of the rice S-like RNases.

Predicted signal peptides are underlined. Residues conserved in other RNase T2 enzymes [5] are shaded. The two conserved active site (CAS) regions are indicated, and the two catalytic histidines are indicated by asterisks.

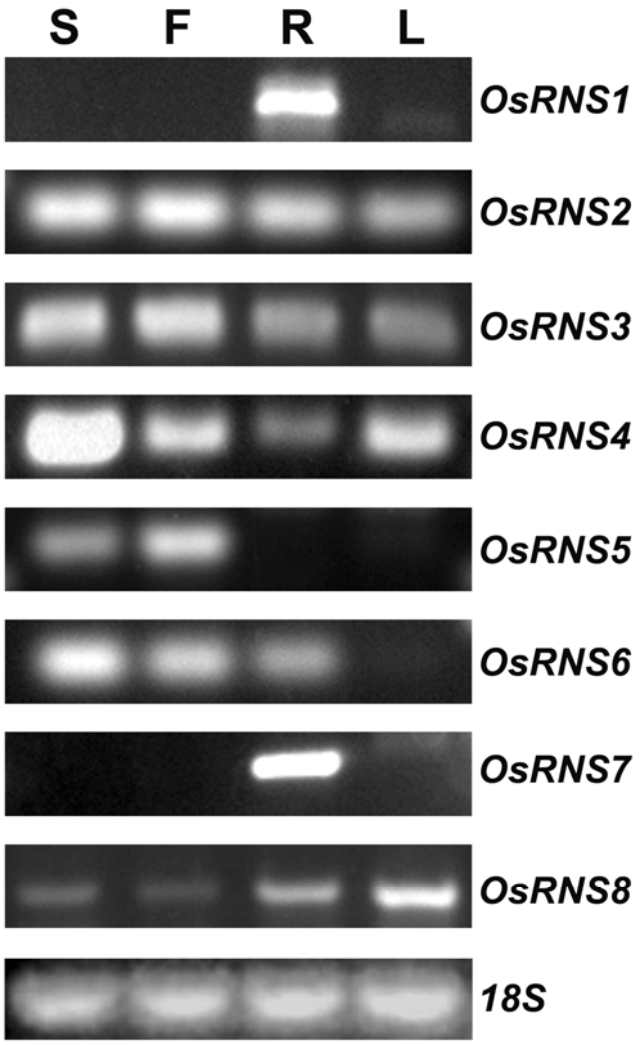


Figure 3 - Expression patterns of the S-like RNase genes from rice.

Total RNA was obtained from stem (S), inflorescence (F), root (R) and leaf (L) tissues of 75-day-old plants, and expression of the eight RNase T2 genes was evaluated by RT-PCR. Amplification of 18S cDNA was used as loading control.

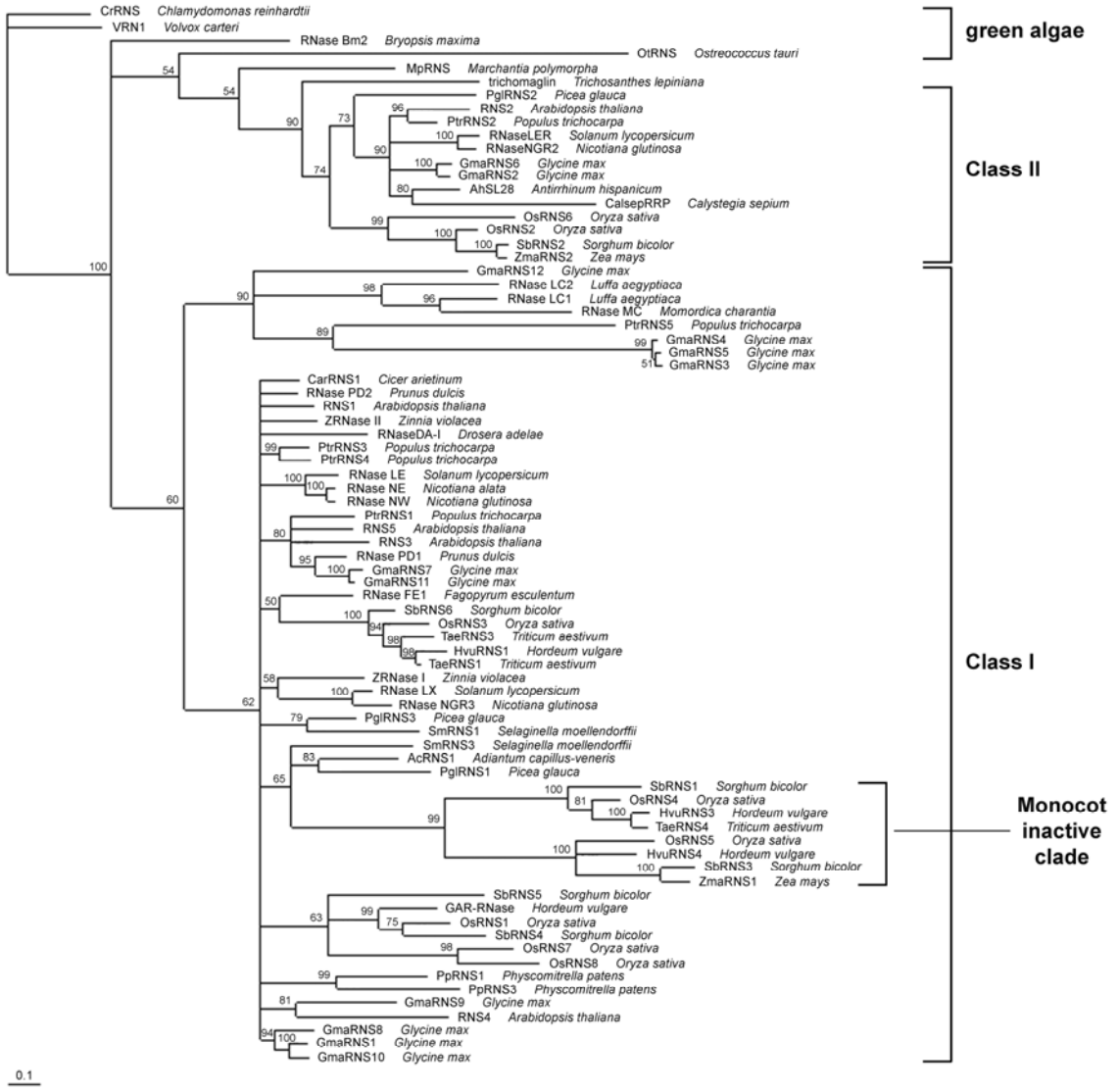


Figure 4 - Phylogenetic relationships of plant S-like RNases.

Tree was estimated by the Bayesian method, using only conserved regions. Bootstrap percentages greater than 50 are shown on interior branches. The tree was rooted using algae sequences. Class I and Class II clades are indicated, as well as algal proteins. The monocot-specific clade that groups inactive S-like RNases (inside Class I) is also labelled.

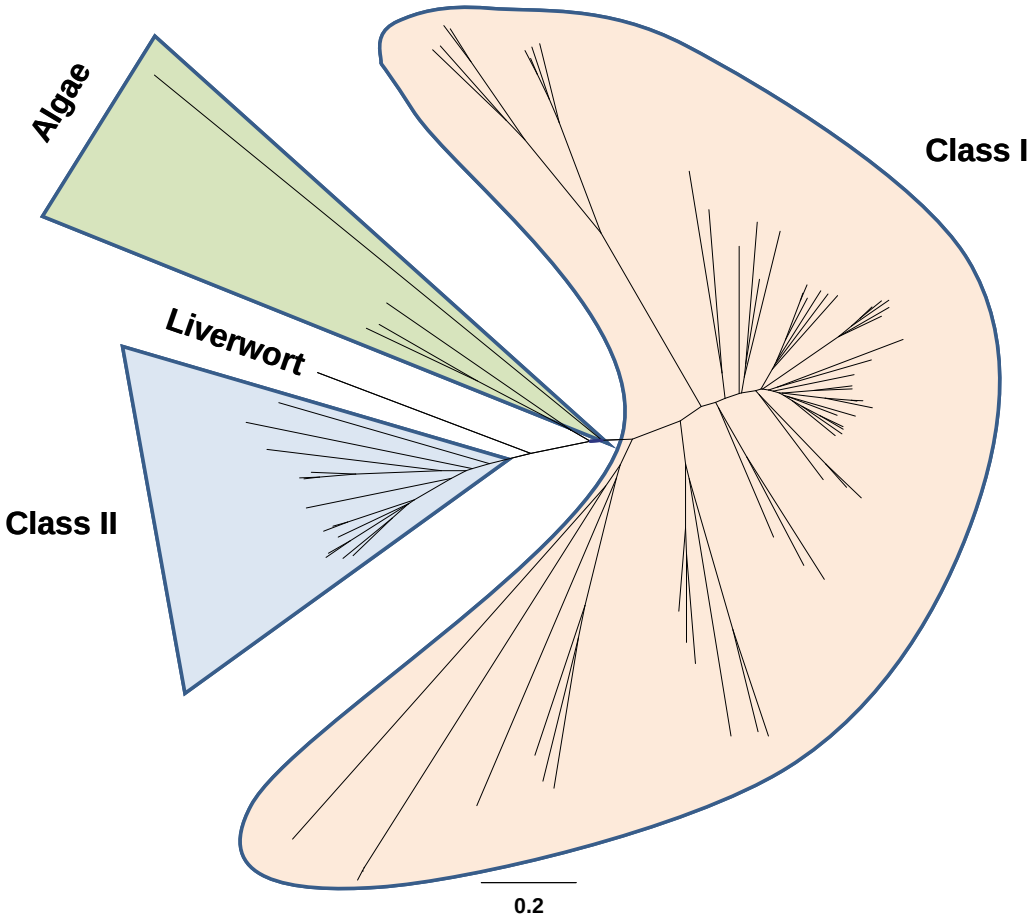


Figure 5 - Phylogenetic tree of plant S-like RNases obtained by the Neighbor-Joining method.

Unrooted tree generated by the NJ method with the same sequences used in Figure 4. Class I (orange), Class II (blue) and algae (green) clusters are shaded. The liverwort (*Marchantia polymorpha*) sequence is also indicated. A detailed, rooted version of this figure, including bootstrap values is shown in Additional file 2.

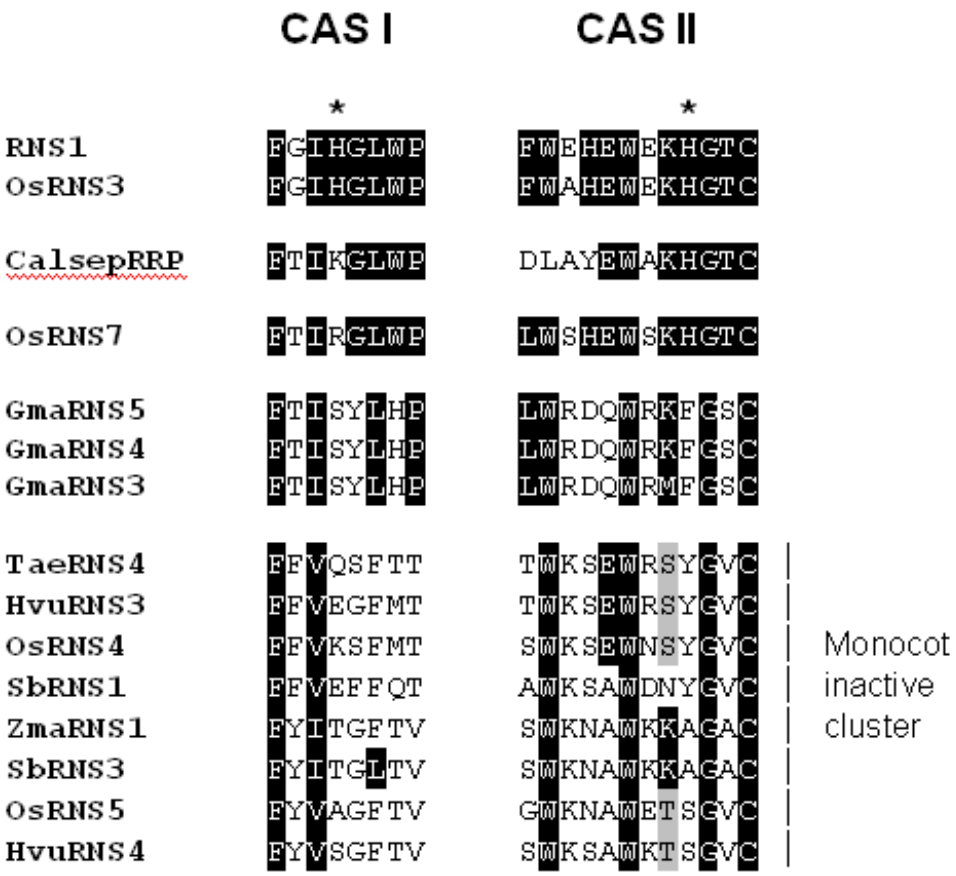


Figure 6 - Mutations in conserved active site residues in plant S-like RNases.

The alignment shows the conserved CAS I and CAS II regions characteristic of RNase T2 enzymes. The catalytic histidines are marked with asterisks. Either one of the two histidines are lost in these proteins, indicating that they are inactive RNases. The active sites of OsRNS3 and RNS1, two active RNases, are shown for comparison. The proteins belonging to the monocot-specific cluster are also indicated.

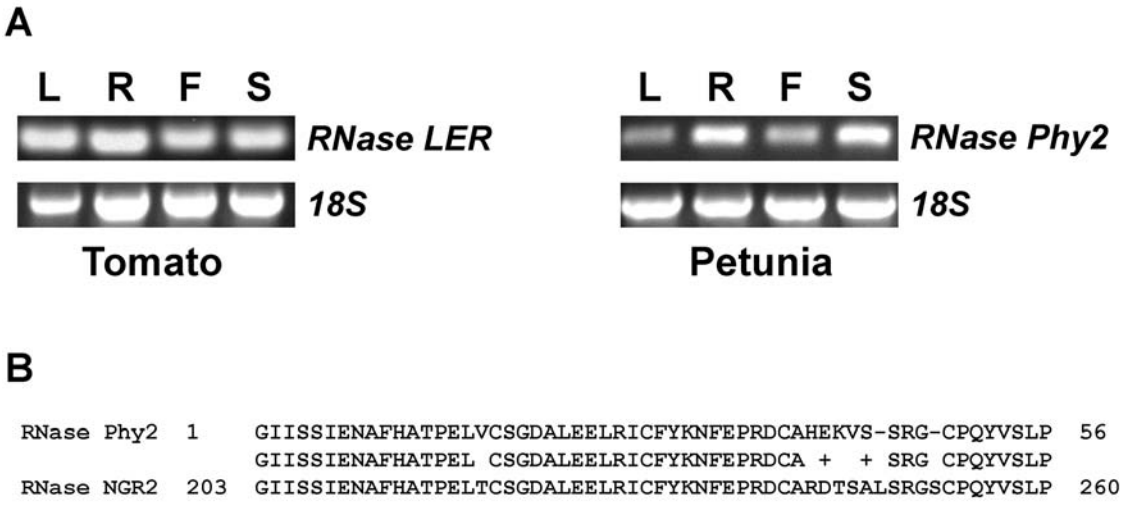


Figure 7 - Conservation and constitutive expression of Class II S-like RNases.

A. RT-PCR analysis of the expression of *RNase LER* from tomato, and a newly identified Class II S-like RNase, *RNase Phy2*, from petunia. Tomato and petunia RNA from stems (S), flowers (F), roots (R) and leaves (L) was analyzed. **B**. Sequence alignment of the predicted peptide encoded by the *RNase Phy2* fragment amplified in A and RNase NGR2, a Class II S-like RNase.

Tables

Gene	MSU Rice Genome Annotation ID	Rice Annotation Project Database (RAP-DB) ID
OsRNS1	LOC_Os07g43670	Os07g0630400
OsRNS2	LOC_Os01g67180	Os01g0897200
OsRNS3	LOC_Os08g33710	Os08g0434100
OsRNS4	LOC_Os09g36680	Os09g0537700
OsRNS5	LOC_Os09g36700	Os09g0538000
OsRNS6	LOC_Os01g67190	Os01g0897300
OsRNS7	LOC_Os07g43600	Os07g0629300
OsRNS8	LOC_Os07g43640	Os07g0629900

Table 1 - Equivalence of our nomenclature and gene identification numbers from the Japanese and US annotations of the rice genome

Gene	Overall expression	Pi-starvation	Other abiotic stress	Biotic stress
OsRNS1	Low	-		
OsRNS2	High – all tissues	-		
OsRNS3	High	-	Drought Salt Cold	
OsRNS4	Variable	-	Wounding Salt	*Xanthomonas oryzae* Beet armyworm Water weevil
OsRNS5	Variable	Root	Wounding Salt	*Xanthomonas oryzae* Beet armyworm
OsRNS6	Low	-		
OsRNS7	Low	Root		*Magnaporthe grisea*
OsRNS8	Low	Root Leaf	Cold	

Table 2 - *OsRNSs* expression data extracted from public databases

Overall gene expression was estimated by the average intensity from microarray data obtained from Genevestigator or number of sequenced tags from the MPSS database. Specific response to biotic and abiotic stresses was also obtained from these databases. Pi-starvation expression was extracted from microarray data [72].

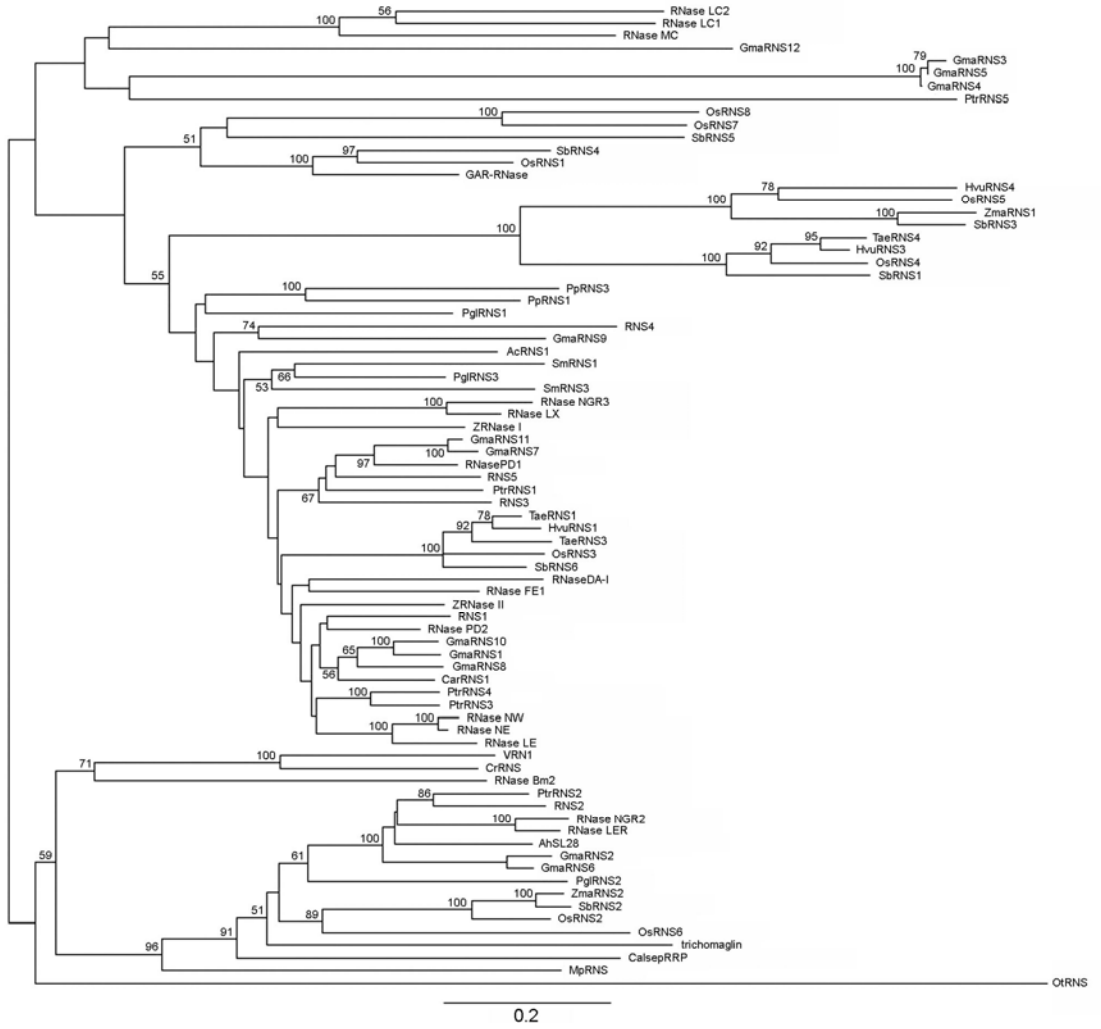
Additional files

Name	Species	Common name	Genome locus	Accesion number
AcRNS1	Adiantum capillus-veneris	maidenhair fern		DK956820
AhSL28	Antirrhinum hispanicum	Spanish Snapdragon		CAC50874
CalsepRRP	Calystegia sepium	hedge bindweed		AAF45022
CarRNS1	Cicer arietinum	chickpea		CAA10130
CrRNS	Chlamydomonas reinhardtii	green alga		XP_001691379
GAR-RNase	Hordeum vulgare	domesticated barley		AAB58718
GmaRNS1	Glycine max	soybean	Glyma02g12010	
GmaRNS10	Glycine max	soybean	Glyma01g05840	
GmaRNS11	Glycine max	soybean	Glyma01g05850	
GmaRNS12	Glycine max	soybean	Glyma03g35230	
GmaRNS2	Glycine max	soybean	Glyma16g03120	
GmaRNS3	Glycine max	soybean	Glyma02g07150	
GmaRNS4	Glycine max	soybean	Glyma02g07140	
GmaRNS5	Glycine max	soybean	Glyma02g07130	
GmaRNS6	Glycine max	soybean	Glyma07g06520	
GmaRNS7	Glycine max	soybean	Glyma02g12020	
GmaRNS8	Glycine max	soybean	Glyma20g04820	
GmaRNS9	Glycine max	soybean	Glyma20g04830	
HvuRNS1	Hordeum vulgare	domesticated barley		AAB58719
HvuRNS3	Hordeum vulgare	domesticated barley		AAM80567
HvuRNS4	Hordeum vulgare	domesticated barley		AAF45043
MpRNS	Marchantia polymorpha	liverwort		BJ850209
OsRNS1	Oryza sativa	rice	Os07g0630400 LOC_Os07g43670	BAF22270
OsRNS2	Oryza sativa	rice	Os01g0897200 LOC_Os01g67180	BAF07000
OsRNS3	Oryza sativa	rice	Os08g0434100 LOC_Os08g33710	BAF23791
OsRNS4	Oryza sativa	rice	Os09g0537700 LOC_Os09g36680	BAF25707
OsRNS5	Oryza sativa	rice	Os09g0538000 LOC_Os09g36700	BAF25708
OsRNS6	Oryza sativa	rice	Os01g0897300 LOC_Os01g67190	BAF07001
OsRNS7	Oryza sativa	rice	Os07g0629300 LOC_Os07g43600	BAC20676
OsRNS8	Oryza sativa	rice	Os07g0629900 LOC_Os07g43640	BAC20680
OtRNS	Ostreococcus tauri	green alga		CAL54360
PglRNS1	Picea glauca	white spruce		EX440507
PglRNS2	Picea glauca	white spruce		EX387106
PglRNS3	Picea glauca	white spruce		EX322444
PpRNS1	Physcomitrella patens	moss	jgi|Phypa1_1|126361|e_gw1.59.205.1	
PpRNS3	Physcomitrella patens	moss	jgi|Phypa1_1|152231|e_gw1.368.41.1	
PtrRNS1	Populus trichocarpa	Black cottonwood		XP_002311303
PtrRNS2	Populus trichocarpa	Black cottonwood		XP_002321228
PtrRNS3	Populus trichocarpa	Black cottonwood		XP_002316136
PtrRNS4	Populus trichocarpa	Black cottonwood		XP_002311302
PtrRNS5	Populus trichocarpa	Black cottonwood		EEE95823
RNase Bm2	Bryopsis maxima	green alga		BAE06157
RNase FE1	Fagopyrum esculentum	common buckwheat		BAF03591
RNase NGR2	Nicotiana glutinosa	tobacco		BAC77612
RNase NGR3	Nicotiana glutinosa	tobacco		BAC77611
RNaseDA-I	Drosera adelae	lance-leawed sundew		BAE16883
RNaseLC1	Luffa aegyptiaca	smooth loofah		BAA10891
RNaseLC2	Luffa aegyptiaca	smooth loofah		BAA10892

RNaseLE	Solanum lycopersicum	tomato		P80022
RNaseLER	**Solanum lycopersicum**	**tomato**		**CAL64053**
RNaseLX	Solanum lycopersicum	tomato		P80196
RNaseMC	**Momordica charantia**	**balsam pear**		**P23540**
RNaseNE	Nicotiana alata	Persian tobacco		AAA21135
RNaseNW	**Nicotiana glutinosa**	**tobacco**		**BAC77813**
RNasePD1	Prunus dulcis	almond		AAG09465
RNasePD2	**Prunus dulcis**	**almond**		**AAF82615**
RNS1	Arabidopsis thaliana	thale cress	AT2G02990	P42813
RNS2	**Arabidopsis thaliana**	**thale cress**	**AT2G39780**	**NP_030524**
RNS3	Arabidopsis thaliana	thale cress	AT1G26820	NP_564264
RNS4	**Arabidopsis thaliana**	**thale cress**	**AT1G14210**	**NP_563940**
RNS5	Arabidopsis thaliana	thale cress	AT1G14220	NP_563941
SbRNS1	**Sorghum bicolor**	**Sorghum**	**Sb02g031160**	
SbRNS2	Sorghum bicolor	Sorghum	Sb03g042630	
SbRNS3	**Sorghum bicolor**	**Sorghum**	**Sb02g031170**	
SbRNS4	Sorghum bicolor	Sorghum	Sb02g040170	
SbRNS5	**Sorghum bicolor**	**Sorghum**	**Sb02g040180**	
SbRNS6	Sorghum bicolor	Sorghum	Sb07g021330	
SmRNS1	**Selaginella moellendorffii**	**spikemoss**	**jgi|Selmo1|75786|e_gw1.0.2678.1**	
SmRNS3	Selaginella moellendorffii	spikemoss	jgi|Selmo1|270532|estExt_fgenesh1_kg.C_60029	
TaeRNS1	**Triticum aestivum**	**bread wheat**		**AAS01727**
TaeRNS3	Triticum aestivum	bread wheat		AAS07016
TaeRNS4	**Triticum aestivum**	**bread wheat**		**AAM18521**
trichomaglin	Trichosanthes lepiniana	Maganlin		1SGL_A
VRN1	**Volvox carteri f. nagariensis**	**green alga**		**BAA95359**
ZmaRNS1	Zea mays	maize		AAB37265
ZmaRNS2	**Zea mays**	**maize**		**ACG36234**
ZRNasel	Zinnia violacea	elegant zinnia		AAC49325
ZRNaseII	**Zinnia violacea**	**elegant zinnia**		**AAC49328**

Additional file 1 – S-like proteins used for phylogenetic analysis

Protein name, species and accession number or locus identification for all the proteins included in our analysis. Proteins with accession number include those obtained from ESTs. Genome locus identification follows Phytozome nomenclature for soybean and sorghum, TAIR nomenclature for Arabidopsis, Joint Genome Initiative (JGI) for mosses, and the two current annotations for rice.



Additional file 2 – NJ phylogenetic tree of plant S-like RNases
Detailed version of the tree depicted in Figure 5. The tree was rooted. Bootstrap percentages (for 1,000 replications) greater than 50 are shown on interior branches.

Gene	Primers	
	Forward	Reverse
OsRNS1	GAATTCGACTTCTTCTACCTC	CAGCTGTTCCAGGTGTACCA
OsRNS2	**GCTCATCCTCTTCCACTTGC**	**ACCACCACTTGCCAACATTT**
OsRNS3	CGCAGGACTACGACTTCTT	ATCTGGGACAGCGTGTAG
OsRNS4	**GGCTTACTTGCGACTTCAGG**	**AAGGATTGTCGGAGTTGC**
OsRNS5	GCTCTGCTCTGCCTTCTTG	ACAGGGAAGAGTTGGTGGTG
OsRNS6	**CCGCTCAAATTCTTCACGAT**	**GAAAGAGCCGCAGTACAAGG**
OsRNS7	GTCGTGTTCTCCCTTGTG	CCGAGCGTGTAGGTCTTC
OsRNS8	**TGCCCAGCAGTGGCCTGAC**	**ATATTGATCGTGTGGCA**
RNase Phy2	GAGGCATCATTTCATCCA	GGCAAGCTGACGTACTGA
RNase LER	**GAGGCATCATTTCATCCA**	**GGCAAGCTGACGTACTGA**

Additional file 3 – Primers used in this work
Primers used for RT-PCR and cloning of rice and petunia RNases

CHAPTER 6: General conclusions

Although function and regulation of T2 RNases have been studied in numerous model systems, plant S-RNases are the only group for which a function is clearly known. These S-RNases are involved in self-incompatibility and are necessary to promote seed diversification from the parent lines. On the contrary, the S-like RNases seem to have alternative functions that are not clearly understood.

S-like RNases are induced during the wounding response, pathogen infection, senescence, phosphate limitation, and abiotic stress treatments just to name a few. Some are active ubiquitously, as is the case with RNS2 and NGR2*;* yet their biological function remains unknown (Taylor, Bariola et al. 1993; Hayashi T 2003). In an attempt to better understand the function of T2 RNases we studied the regulation of *RNS1* in *Arabidopsis*. *RNS1* is induced both locally and systemically when the leaves are wounded; yet this expression is independent of the known wounding pathways and hormone jasmonic acid. Thus RNS1 is part of a novel wounding response pathway making it a desirable candidate for studying gene function and regulation.

The promoter of *RNS1* contains many stress response elements such as WUN, W-Box, MYB, MYC, DRE, and ABRE sites (Chapter 2). These elements suggest *RNS1* is a highly regulated gene and necessary for more than one type of stress response. The hormone ABA is also regulated by abiotic stress. As shown in Chapter 2, *RNS1* is induced by exogenous ABA application; and mutants in this pathway have a reduction of the *RNS1* transcript when wounded. The induction of *RNS1* by ABA and not by jasmonic acid is evidence supporting a unique role of RNS1 in the wound response.

To further study the function of T2 RNases we identified four T2 RNases in *Petunia hybrida* (Chapter 3). Of these four RNases, two show high similarity to known S-like RNases (*RNase Phy1* and *RNase Phy5*); however the other two (*RNase Phy3* and *RNase Phy4*) have properties of both S- and S-like RNases. The

expression patterns of these particular RNases (*Phy3* and *Phy4*) suggest they may have a role in nectar defense against microorganisms.

In addition to studying their function and regulation, we investigated the evolution of the T2 RNase family. These RNases are highly intriguing because they are conserved in both plants and animals. We chose to use *Danio rerio* (zebrafish) to study both regulation and evolution of these RNases in animals (Chapter 4). Zebrafish contain two T2 RNases, one fish specific (RNase DRE1 clade) and one common to all chordates (RNase DRE2 clade). Expression of *RNase DRE2* in all tissues suggests a role as a housekeeping enzyme. Analysis of evolution in the T2 RNase family in plants and animals suggests the emergence of the RNase A family in vertebrates influenced the evolution of the T2 family in this group.

Evolution of the T2 family in eukaryotes is very complex, not only because of the emergence of the RNase A family in animals but also due to gene duplication occurring in plants. Gene duplication often leads to paralogs of gene families, as seen in Arabidopsis which contains 5 S-like RNases. Additionally, *Oryza sativa* (rice) contains 8 copies of T2 RNases that are divided into two classes (Chapter 5). Class I T2 RNases are highly diverse and thought to have undergone subfunctionalization, whereas class II enzymes are hypothesized to have a role in a housekeeping function. Plant class II RNases resemble the animal RNase T2 enzymes in evolutionary history and expression patterns, and could also have a housekeeping role, while plant Class I has an evolutionary history similar to that observed for RNase A in vertebrates, and the proposed functions for these RNases are also similar.

Conservation of T2 RNases throughout plants and animals suggests these RNases have important roles in both cellular maintenance in all organisms, and stress responses in plants. Our work identified unique regulation for these enzymes, and also advances in our understanding of their potential biological functions, although more work is still needed to fully understand their role in different organisms.

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APPENDIX - Regulation and function of *RNS1* expression

Introduction

During transcription, a cell has numerous transcription factors positively enforcing the upregulation of specific necessary transcripts at a given time. Although countless transcription factors have been identified, most of the genes that each one regulates are not known.

During stress responses, transcription factors that regulate defense, recycling, and recovery may be activated; and each stress response may yield a new combination of these factors for that specific response. For example, during the wounding response WUN and W-BOX elements are recognized by a set of transcription factors and during salt and drought stress ABRE, MYB, MYC, and DRE elements are recognized by a different set of transcription factors (Van Buskirk and Thomashow 2006). Without the specific interaction between the promoter elements and transcription factors the plant would not be able to respond to the stress and alter the regulation of its cellular processes.

In Chapter 2 we showed that regulation of *RNS1* by wounding is independent of the common signaling pathways that control the plant response to this stimulus. We also showed that ABA participates in the regulation of *RNS1* expression; however, the best characterized transcription factors that regulate ABA-dependent expression did not control *RNS1* expression. The promoter region of *RNS1* has many of the above mentioned transcription factor element binding sites. These include WUN, MYB, MYC, DREB, and ABRE elements (Chapter 2, Supplemental Figure S1). Analysis of RNS1 promoter-GUS fusion transgenic plants led us to identify potential candidate transcription factors that could regulate *RNS1* expression. Here we analyze the participation of some of those transcription factors in the regulation of RNS1 expression, as well as other components of the ABA signal transduction pathway.

Expression of RNS1 in response to wounding suggests that this enzyme participates in a defense mechanism, although it is not clear what the specific

biological function of RNS1 is. In order to better understand the function of RNS1 we investigated the effect of disrupting the expression of RNS1 on plant defenses, and attempted to identify the subcellular localization of the enzyme.

ABRE transcription factor

Abiotic stresses such as drought, salinity, and low temperatures induce production of ABA (Koornneef et al. 1998, Nishimura et al. 2005). Recently much work has uncovered transcription factors associated with ABA regulation. One possible candidate for ABA regulation of *RNS1* is AREB1 (Abscisic Acid Responsive Elements-Binding protein - also known as ABF2 – AREB Binding Factor). ABF2 is an ABA responsive element (ABRE) binding factor (ABF). This protein is a leucine zipper-type transcription factor, binding to a consensus sequence in the promoter region of some ABA regulated genes (Uno et al. 2000). Analysis of the promoter region of *RNS1* detected 3 recognition sites for ABRE binding elements (Chapter 2, Supplemental Figure S1). To determine if ABF2 can regulate the expression of *RNS1* during the wounding response we tested the *abf2* mutant in a wounding experiment (as described for ABI3, ABI4 and ABI5 in Chapter2, Methods). Northern blot analysis of *RNS1* in wounded *abf2* plants did not differ significantly from the signal detected in wild-type control plants (Figure 1). Therefore we concluded that ABF2 is not the ABRE-binding transcription factor regulating the ABA-dependent expression of *RNS1* during the wounding response.

PHR1 transcription factor

The transcription factor PHR1 participates in the induction of *RNS1* during phosphate limitation according to Rubio et al (2001). Previously, it was known that under phosphate limiting conditions, *RNS1* is induced (Bariola et al. 1994) however the transcription factors responsible for this induction were unknown. This induction is affected by the MYB transcription factor PHR1 (Rubio et al. 2001). PHR1 acts as a central factor that contributes to downstream phosphate (Pi) signaling by regulating the expression of a wide range of Pi-responsive genes (Rubio et al.

2001). *phr1* mutants grown in phosphate limiting conditions have a reduction in the amount of *RNS1* transcript compared to wild-type (Rubio et al. 2001). To determine if this transcription factor is necessary for induction of *RNS1* expression during wounding, *phr1* mutants were assayed by wounding experiments similar to those done to analyze the role of ABF2. Northern blot analysis showed no difference in induction of the *RNS1* transcript under wounding conditions in the *phr1* mutants (Figure 2). Thus regulation of *RNS1* during wounding is independent of the *PHR1* MYB transcription factor.

ABH1 post-transcriptional regulator

In addition to transcriptional control, posttranscriptional regulation can also affect the steady-state level of mRNAs that accumulate in a cell. Several proteins associated with ABA signaling also affect RNA metabolism. Characterization of ABA-hypersensitive mutants showed that the genes affected corresponded to RNA binding proteins (RBP), including ABH1, HYL1, and SAD1. *ABH1* codes for a mRNA cap binding protein and participates in early ABA signal transduction (Hugouvieux et al. 2001). It binds to the 5' end of ABA regulated transcripts, and it is possible it may bind to the 5'end of the *RNS1* transcript because this transcript is upregulated by ABA treatments. Wild-type and *abh1* plants were grown, wounded, and analyzed according to the methods used in Chapter 2. *RNS1* mRNA accumulates to about 50% of the level observed in WT in *abh1* plants in response to wounding. A similar effect is observed in response to ABA treatments (Figure 3). Therefore, ABH1 activity is required for normal *RNS1* accumulation. To determine if the effect of ABH1 is also observed at the protein level, we chose to do an *in gel* assay in which we can quantify the activity of RNS1 as an indirect measurement of protein accumulation. Samples were taken from control and *abh1* plants treated with ABA or wounded for 12 or 24 hours. Proteins were then extracted according to Chapter 2 (Methods) and used for the *in gel* RNase Activity Assay. Equal amounts of protein were loaded in all the lanes as determined by a Bradford assay.

Differences in RNS1 activity are shown in Figure 4. RNS1 activity is consistently lower in the *abh1* mutant compared to the wild-type control plants.

Pathogen infection

Pathogen infection in tobacco plants induces two different T2 RNases, *NGR3* during TMV infection and *RNaseNE* during *P parasitica* (Galiana et al. 1997, Kurata et al. 2002). Expression analysis of *RNS1* during wounding treatment suggests this ribonuclease may also play a role in plant defense responses. In fact, microarray data from the TAIR database supports the idea that *RNS1* is induced during plant infection by *Pseudomonas syringae DC3000* and is significant at 24 hours post infection (Figure 5). Inoculation damage to the local leaves is noted by the increase in *RNS1* signal in the mock sample; however mock-treated plants have a significantly lower level of *RNS1* then plants treated with DC3000 or and *P. syringae* pv. phaseolicola (non-host resistant) in early infection. DC3000-treated plants accumulate more *RNS1* transcript at 24 hours of infection than mock or phaseolicola-treated plants. This suggests that RNS1 is involved in defense responses against bacterial infections.

Next, we tested viral infection in *rns1* knockout and overexpressor plants using Turnip Mosaic Virus (TuMV), and Cucumber Mosaic Virus (CMV). Plants were infected with a GFP tagged variant of TuMV (Whitham et al. 2003) and the rate of infection was monitored for 15 days. Infection was detected in the apical flowers and in the inoculated leaves in all plant lines (Figure 7A). However after 3 replicate experiments there were no differences in rates of infection. Additionally, tissue samples were collected from either TuMV or CMV infected plants at set time points and used for northern blot analysis. Results from data analyses showed no induction of *RNS1* in the control plants; the only detectable expression was in the RNS1 overexpressing line (Figure 7B). Thus *RNS1* is not induced nor does it appears to have a role in TuMV or CMV infection in *Arabidopsis*; however this does

not mean that RNS1 is not involved in response and protection against other viruses.

Aphid infestation on *rns1* plants

Wounding of *Arabidopsis* plants induces *RNS1* expression. Although aphids are phloem feeders, they must still penetrate through some cells and the extracellular space to reach the phloem. This penetration causes minimal damage to local cells; yet damage does occur. Because RNS1 is hypothesized to be extracellular and it is induced during the wounding response, we wanted to determine if RNS1 has a role in plant-aphid interaction. We challenged wild-type, *rns1*, and RNS1 ox plants with the aphid *Myzus persicae* (also known as the Green Peach Aphid). Four to five week old plants with bolts approximately 2.5-4 cm were infested with 8 aphids per plant. After 7 days of aphid infestation, aphids were counted to determine if RNS1 had an effect on aphid growth (Figure 8). No difference in aphid performance was detected, concluding RNS1 is not required for plant defense against aphids.

RNS1 localization

Apoplast isolation

To gain a better understanding of RNS1 function, we can also look at the localization of RNS1. RNS1 contains a secretion signal peptide; however the final location of RNS1 remains unknown. Early *Arabidopsis* cell culture work using the *in gel* assay of proteins isolated from cells or proteins isolated from media suggest that RNS1 is secreted into the media (Bariola et al. 1999). To determine if RNS1 is secreted to the space between the cell membrane and cell wall or the extracellular space we chose to isolate proteins from the apoplast. Preliminary results from wounded plants show an enrichment of RNS1 in the wounded apoplast sample compared to the control sample (Figure 9). RNS1 activity is still present in the tissue portion of the wounded sample; however the amount of activity relative to the total amount of protein supports the hypothesis that RNS1 is enriched in the apoplast

sample. Thus it is possible RNS1 is an extracellular protein which is secreted from the cell into a region of the plant where RNA is not normally thought to exist.

CFP tagged RNS1

Preliminary analysis of apoplast proteins suggest RNS1 is secreted into the extracellular space; however the location of this protein in the apoplast remains unknown. To further study the localization of RNS1 we transformed plants with a 35S:RNS1:CFP construct (hygromycin resistant). Mutant seedlings containing the appropriate resistance marker were identified and moved to individual pots. Next tissue from the resistant plants was screened for a change in RNase activity by the *in gel* assay. Only plants expressing a functional RNase with an appropriate molecular weight (approximately 45kDa) were selected for microscopy. However, this rigorous selection process does not ensure a correctly folded CFP molecule, nor the amount of detection observed under a confocal microscope. Preliminary results using CFP control plants suggest there are equipment problems or CFP stability problems in detecting our version of CFP (GC MacIntosh, A Meyer, M Hillwig, unpublished); therefore we are unable to confirm the location of the RNS1-CFP construct in our transgenic plant lines.

Conclusions

As stated in Chapter 2; *RNS1* is induced during wounding and ABA application. After many attempts at unraveling the complex regulation of RNS1, we found that ABH1 participates in the regulation of RNS1 expression as an intermediate in the ABA-dependent pathway. However, we are still perplexed that none of the transcription factors known to regulate ABA responses and even some known to affect RNS1 expression in response to other stimuli do not mediate the ABA regulation of RNS1. Although expression analyses suggest that *RNS1* has an important function during the wounding response, its regulation seems far more complicated than we anticipated. By determining the regulation and location of

RNS1, it may be possible to determine the function of *RNS1* as part of the wounding response and ABA pathways.

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Figures

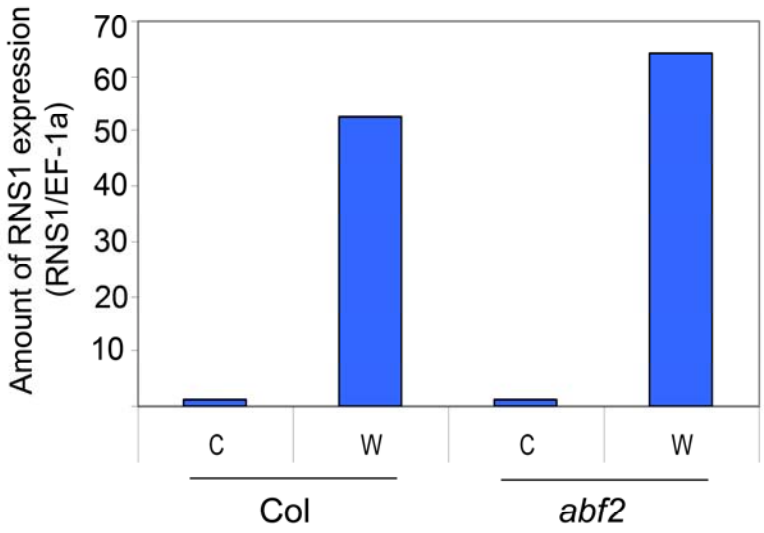

Figure 1 RNS1 expression in *abf2*; an ABRE mutant. Northern analysis of RNA isolated from wounded 4 week old plants. The *EF-1α* probe was used as a control for loading. Col, Columbia (wild-type)

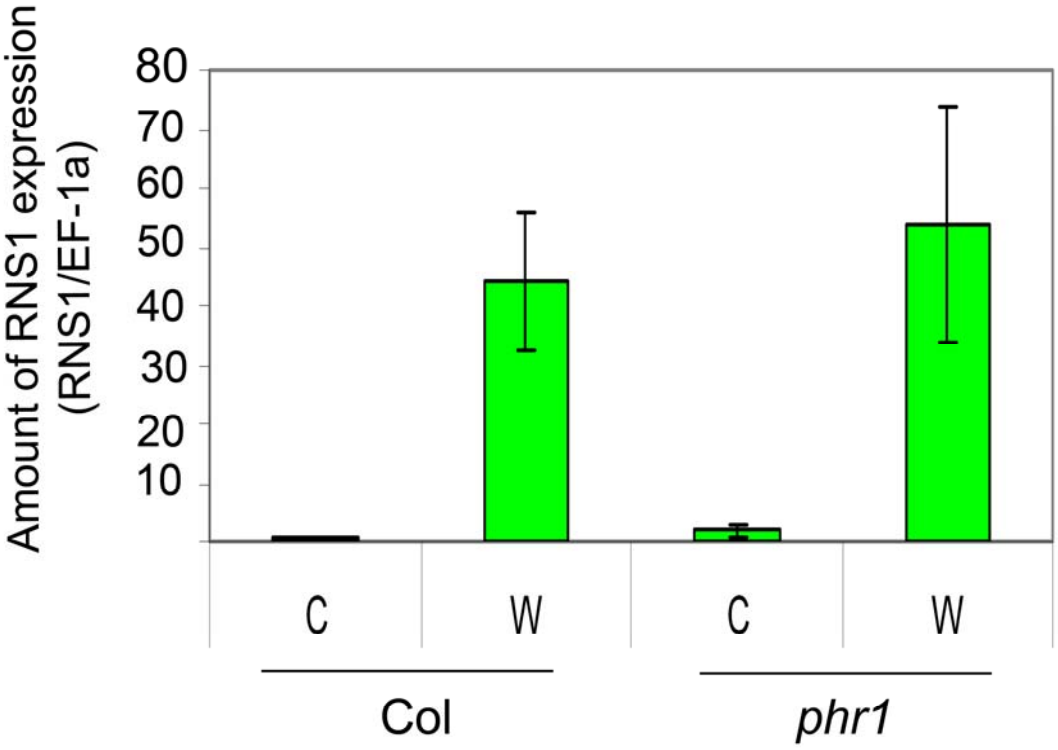

Figure 2 PHR1 does not affect *RNS1* in wounded plants. Northern blot analysis of RNA isolated from wild type (Col) and the mutant *phr1* adult plants wounded for 4 hours. The *EF-1α* probe was used as a control for loading.

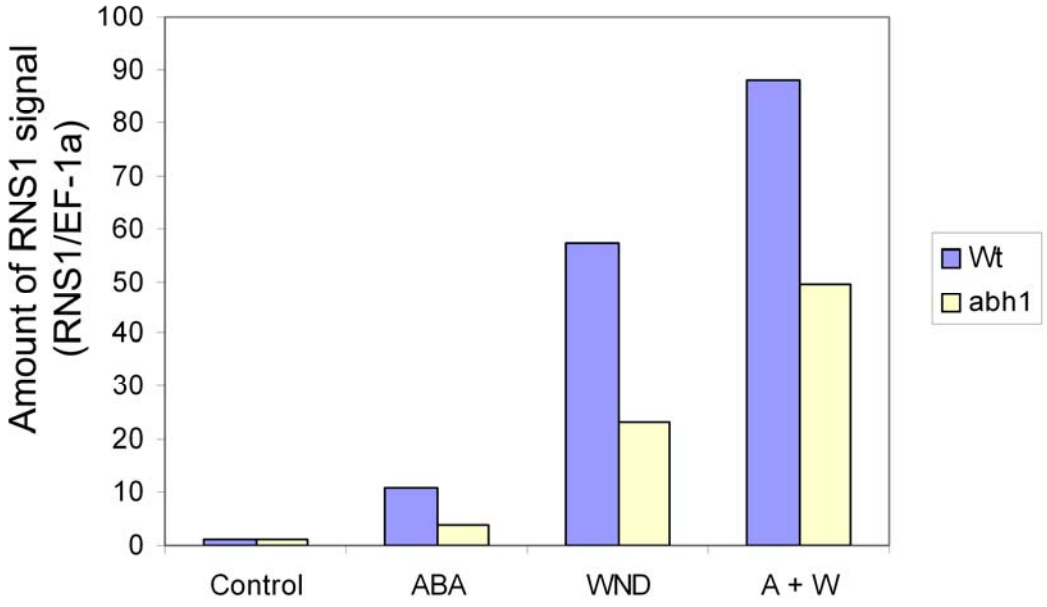

Figure 3 Decrease in *RNS1* expression in the *abh1* mutant. Northern blot analysis of RNA isolated from wild type control and the *abh1* mutant plants treated with ABA, wounded for 4 hours, or both ABA and wounding treatments. The *EF-1α* probe was used as a control for loading.

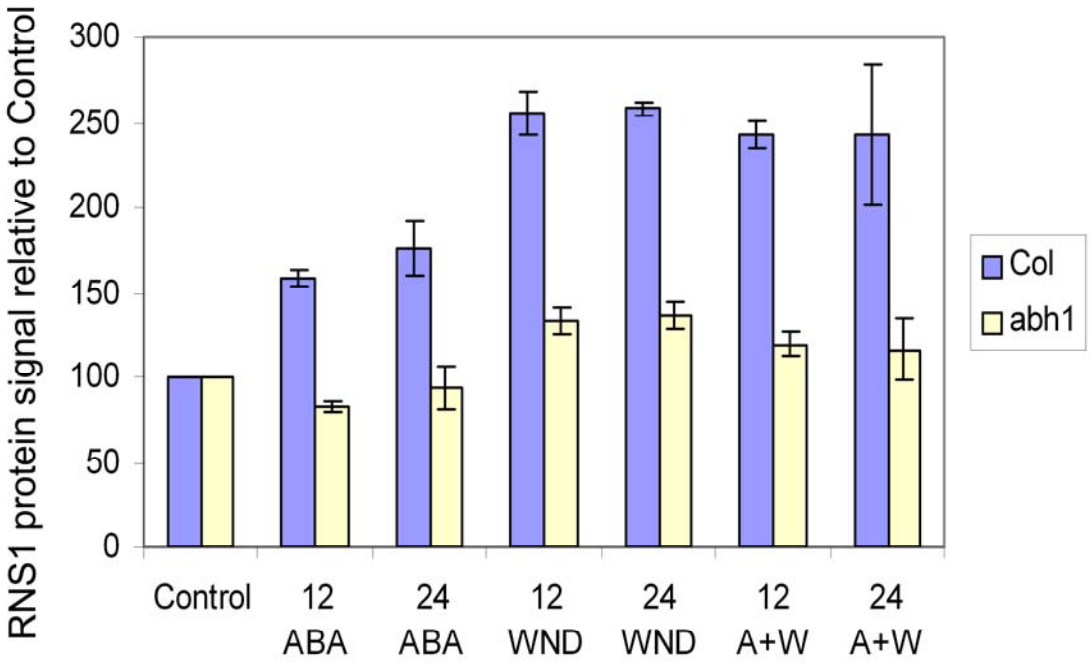

Figure 4 Effect of ABH1 regulation on RNS1 protein expression. Protein was isolated from wild type and *abh1* mutants after the designated time points. Plants were treated with either ABA, wounded, or a combination of both treatments and frozen immediately in liquid nitrogen. Proteins were extracted, quantified, and 20µg was run on an RNase Activity gel. Protein amounts were quantified relative to the signal detected in the control (no treatment) lane for each plant line.

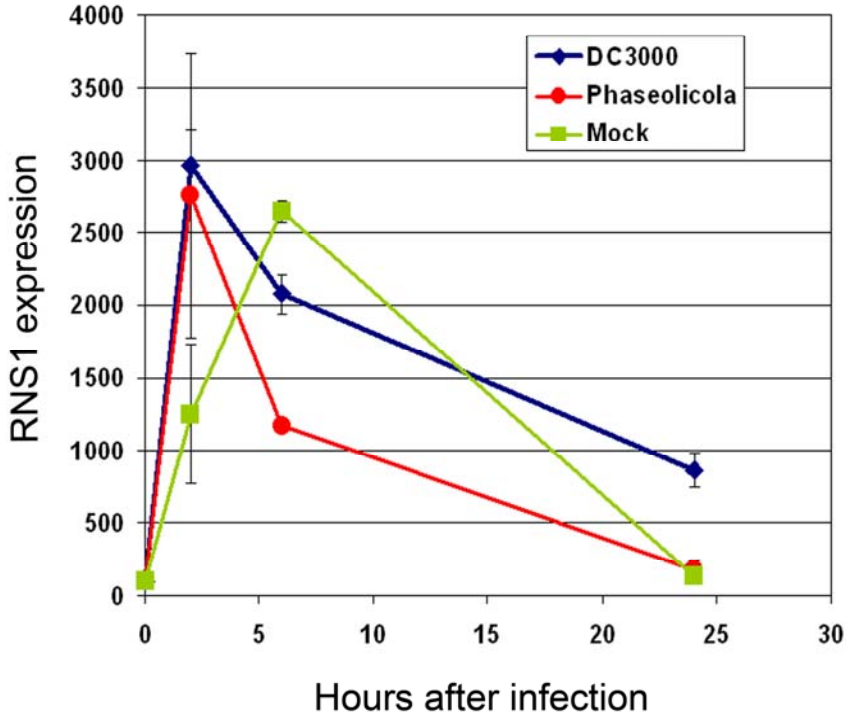

Figure 5 *Pseudomonas syringae* infection on *Arabidopsis* induces *RNS1*. Microarray results of plants treated with a virulent, non-host, and mock infection show *RNS1* is induced rapidly induced in *P syringae* compared to the mock treatment. Induction of *RNS1* does occur in the mock treatment due to manipulation; however this effect occurs after initial infection by the bacteria and is diminished within 24 hours of treatment.

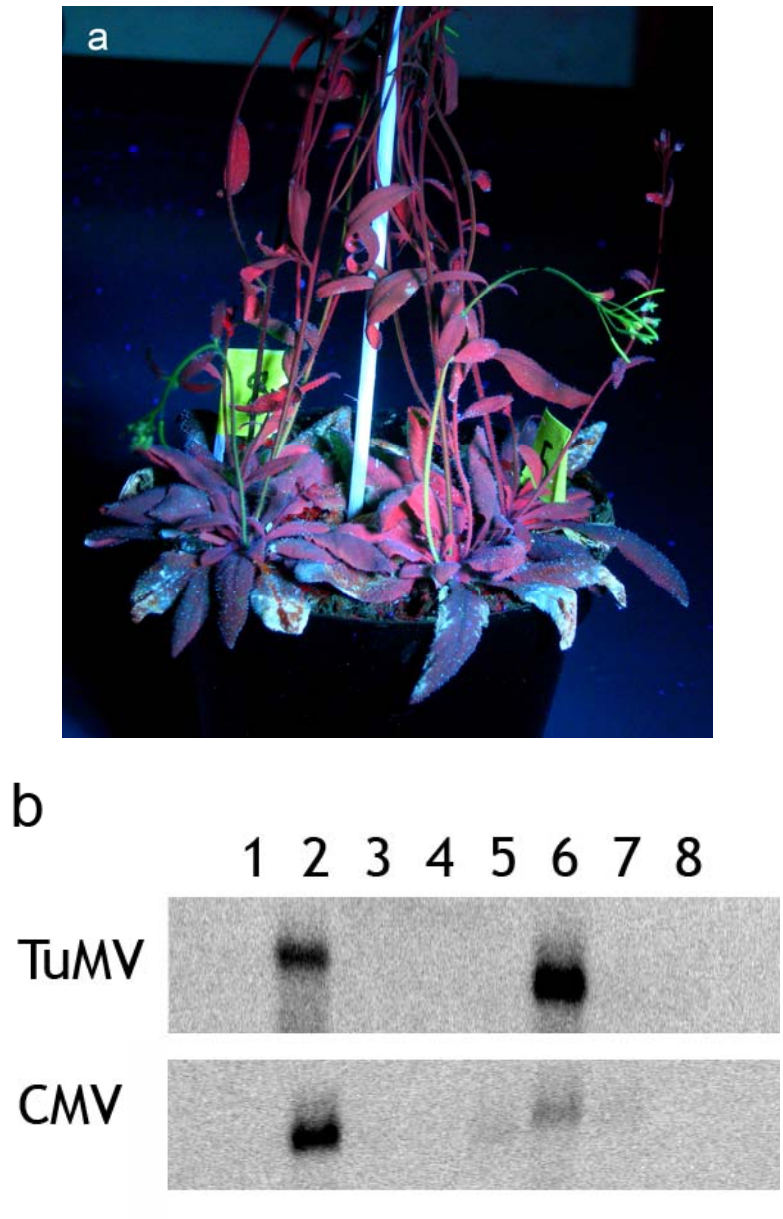

Figure 6 *RNS1* had no effect on virus infection when treating *Arabidopsis* with TuMV or CMV. **(a)** Infection with TuMV, depicted by the green fluorescence, progressed at relatively the same time in wild type, *rns1* mutant, and *RNS1* overexpressing plants. A representative picture showing TuMV infection is shown. (b) Tissue was collected from infected plants 2 days after inoculation. mRNA was hybridized and probed with *RNS1*.
Lane 1 – Col local; lane 2 - *RNS1ox* local; lane 3 – Ws local; lane 4 – *rns1* local; lane 5 – Col systemic (syst); lane 6 – *RNS1ox* syst; lane 7 – Ws syst; lane 8 – *rns1* syst

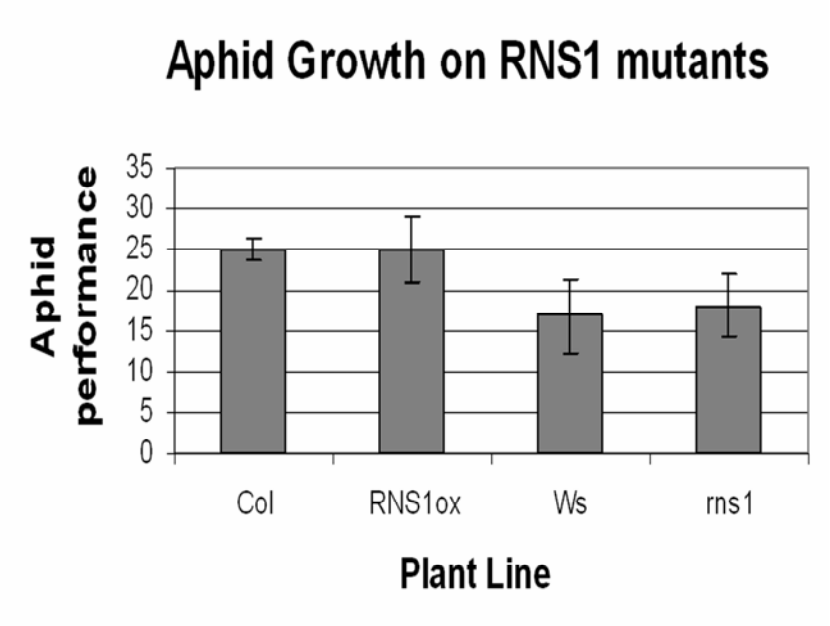

Figure 7 RNS1 has no effect on aphid performance. *RNS1ox* and *rns1* mutants were grown along with their respective controls for 3-4 weeks. Aphid growth was measured by infesting a plant with 8 aphids. Plants were grown with four per pot and each infested. Each experiment had a minimum of 3 pots per line and was repeated at least twice. Aphids were counted after 7 days of infestation and reported as an average per pot per experiment.

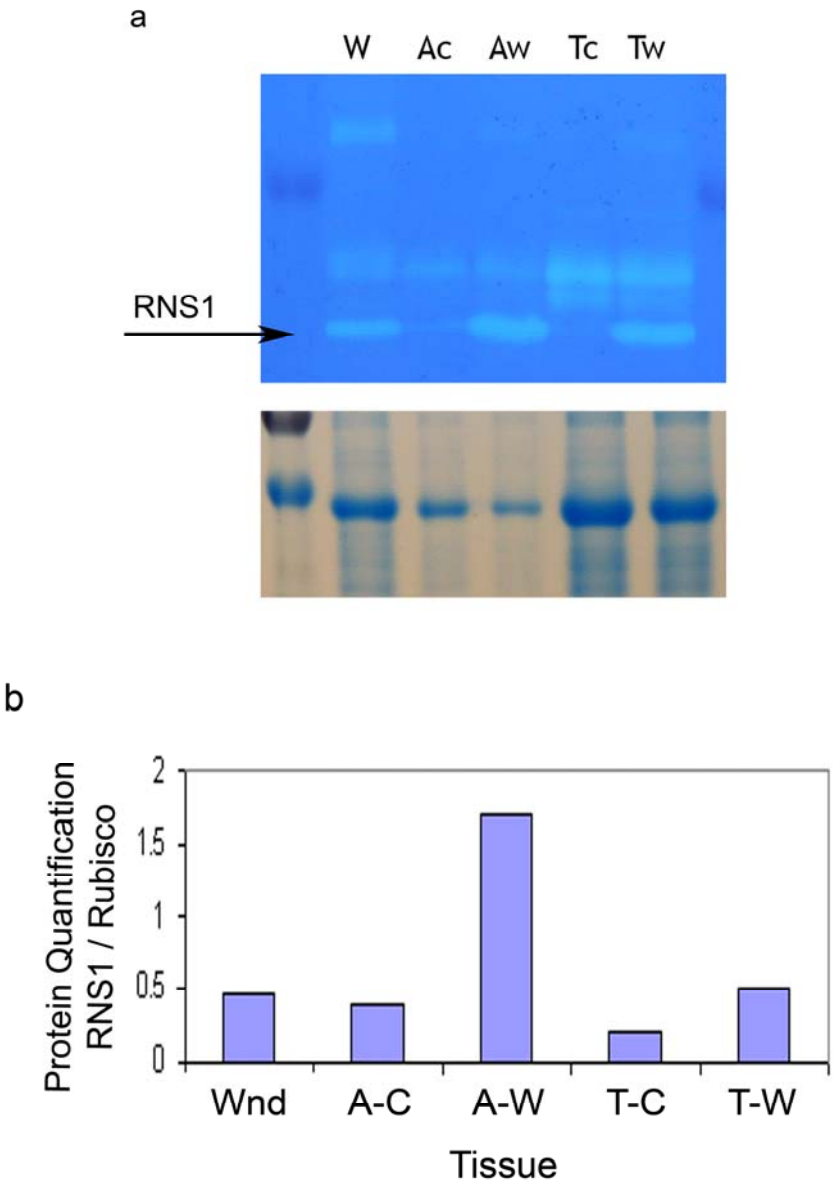

Figure 8 RNS1 activity is detected in apoplast protein samples. 4 week old Col plants were wounded for 24 hours or left alone as control samples. Tissue was then collected and vacuum infiltrated for 3 min using protein extraction buffer with protease inhibitors. The buffer was then used for apoplast samples and the remaining tissue was frozen and residue proteins were extracted for the tissue samples. Order on gel: Wnd – 12 hour wounding leaf sample without apoplast protein extraction; AC – apoplast proteins from control leaves; AW – apoplast proteins from local wounded leaves; TC – tissue from control apoplast sample; TW – tissue from wounded apoplast sample.

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